E-selectin-Monocyte Interactions Facilitate Pulmonary Metastasis

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SUMMARY

Metastasis is a multistep process depending on complex interactions between tumor cells and the stromal compartment which also contains immune cells and soluble factors such as chemokines. The presence of certain classes of monocytes in malignant tumors is frequently a result of chemoattractants such as CCL2 and correlates with enhanced tumor cell extravasation and metastasis. Selectins are vascular adhesion receptors, well-known to facilitate metastasis by mediating the contact between tumor cells and cells in the metastatic environment. Particularly E-selectin is thought to mediate the arrest of tumor cells in the vasculature by binding to E-selectin ligands on tumor cells and thereby promoting metastasis. This study aimed to elucidate whether E-selectin facilitates metastasis by interacting with other constituents of the metastatic microenvironment.

Experimental metastasis of tumor cells without E-selectin ligands to the lungs was attenuated in E-selectin deficient mice. This phenotype can already be determined during early metastatic phases. Lung analysis within the first hours after tumor cell injection revealed reduced expression of endothelial activation markers and the chemokine CCL2 in E-selectin deficient mice. Endothelial activation includes E-selectin up-regulation in C57BL/6 mouse lungs and was shown to be partially dependent on tumor cell-derived CCL2. However, endothelial cells and recruited monocytes were identified as the main sources of CCL2 in the metastatic lungs. The total pool of CCL2 in metastatic lungs was decreased in the absence of E-selectin. This is linked to the reduced leukocyte infiltration observed in E-selectin deficient lungs upon tumor cell injection. Association of infiltrated leukocytes with tumor cells was E-selectin dependent. Moreover, monocyte-supported tumor cell transmigration required binding of E-selectin to monocyte ligands. In the presence of E-selectin, tumor cells together with monocytes induced endothelial cell retraction. Accordingly, the lung vasculature was less permeable in E-selectin deficient or monocyte-depleted mice after tumor cell injection. E-selectin deficient mice also showed decreased spontaneous lung metastasis. Our study identifies a novel mechanism how E-selectin-leukocyte interactions supported by CCL2 induce vascular permeability, which promotes tumor cell extravasation and metastasis.

ZUSAMMENFASSUNG

Die Metastasierung von Tumoren ist ein mehrstufiger Prozess, welcher von komplexen Interaktionen zwischen Tumorzellen und dem Stroma, wie zum Beispiel Immunzellen und Chemokinen abhängt. Das Chemokin CCL2 ist in Primärtumoren präsent und zieht Monozyten an, deren Anzahl in malignen Tumoren häufig mit erhöhter Extravasation von Tumorzellen und dem Auftreten von Metastasen korreliert. Selectine sind vaskuläre Adhäsionsmoleküle und bekannt dafür die Metastasierung zu fördern, indem sie den Kontakt zwischen Tumorzellen und endogenen Bestandteilen ihrer Umgebung ermöglichen. Es wird vermutet, dass besonders E-selectin die Metastasierung begünstigt durch die Vermittlung der Anhaftung von Tumorzellen an die Vaskulatur. Diese Studie hatte das Ziel herauszufinden ob E-selectin die Metastasierung unterstützt, indem es auch mit anderen Komponenten des metastatischen Umfeldes interagiert.

Die Metastasierung von Tumorzellen ohne E-selectin Liganden zur Lunge war verringert in E-selectin-defizienten Mäusen in experimentellen Metastasenmodellen. Dieser Phänotyp war schon während frühen metastatischen Phasen erkennbar. Die Analyse von Lungengewebe während der ersten Stunden nach Tumorzellinjektion zeigte reduzierte Expression von endothelialen Markeren und dem Chemokin CCL2 in E-selectin-defizienten Mäusen. Endotheliale Aktivierung beinhaltet die Hochregulierung von E-selectin in Lungen von C57BL/6 Mäusen was teilweise vom Tumorzell-stammenden CCL2 abhängig war. Endothelzellen und inflammatorische Monozyten wurden jedoch als die wichtigere Quelle von CCL2 in der metastatischen Lunge identifiziert. Der Gesamtpool von CCL2 in der metastatischen Lunge war reduziert in Abwesenheit von E-selectin. Dies stand im Zusammenhang mit der reduzierten Infiltration von Leukozyten in die Lungen von Mäusen ohne E-selectin nach Tumorzellinjektion. Die Assoziation von infiltrierten Leukozyten mit Tumorzellen war E-selectin abhängig und die Monozyten-unterstützte Tumorzell-Transmigration benötigte das Binden von E-selectin an Liganden von Monozyten. Tumorzellen und Monozyten zusammen lösten die Retraktion von E-selectin exprimierenden Endothelzellen aus. Demzufolge war die Lungenvaskulatur weniger durchlässig in Mäusen

ohne E-selectin oder nach Depletion von Monozyten vor der Tumorzellinjektion. E-selectin-defiziente Mäuse wiesen auch weniger spontane Metastasierung in der Lunge auf. Unsere Studie identifizierte einen neuen Mechanismus der beschreibt wie die Interaktion zwischen E-selectin und Leukozyten, unterstützt durch CCL2, vaskuläre Permeabilität erzeugt und dadurch die Extravasation von Tumorzellen und Metastasierung begünstigt.

LIST OF ABBREVIATIONS

CAFs:	cancer associated fibroblasts
CCL2 (MCP-1):	CC chemokine 2 (monocyte chemoattractant protein-1)
CCR2:	CC chemokine receptor 2
CD:	cluster of differentiation
DCs:	dendritic cells
DR3:	death receptor 3
ECM:	extracellular matrix
EGF:	epidermal growth factor
EMT:	epithelial-to-mesenchymal transition
ERK:	extracellular-signal regulated kinase
ESL-1:	E-selectin ligand-1
FGF:	fibroblast growth factor
G-CSF:	granulocyte-colony stimulating factor
GlyCAM-1:	glycosylation-dependent cell adhesion molecule-1
HUVECs:	human umbilical vein endothelial cells
ICAM-1:	intracellular adhesion molecule-1
IFN-γ:	interferon-γ
IL:	interleukin
iNOS:	inducible nitric oxide synthase
JNK:	c-Jun-N-terminal kinase
LOX:	lysyl oxidase
M1/N1:	pro-inflammatory macrophages/neutrophils, M1/N1 phenotype
M2/N2:	pro-tumor macrophages/neutrophils, M2/N2 phenotype
MAdCAM-1:	mucosal addressin cell adhesion molecule-1
MAPK:	mitogen-activated protein kinase
M-CSF/CSF-1:	(macrophage-) colony stimulating factor(-1)
MDSCs:	myeloid-derived suppressor cells

MHC:	major histocompatibility complex
MMP:	matrix metalloproteinase
NF-κB:	nuclear factor kappa B
NK cell:	natural killer cell
PDGF:	platelet-derived growth factor
PI3K:	phosphatidylinositol 3 kinase
PLC:	phospholipase C
PNAd:	peripheral lymph node addressin
PSGL-1:	P-selectin glycoprotein ligand-1
ROS:	reactive oxygen species
SCID:	severe combined immunodeficiency
SDF-1:	stromal derived factor-1
$sLe^{x/a}$:	sialyl-$Lewis^{x/a}$
STAT3:	signal transducer and activator of transcription 3
TAMs:	tumor associated macrophages
TANs:	tumor associated neutrophils
TF:	tissue factor
TGF-β:	transforming growth factor-β
TME:	tumor microenvironment
TNF-α:	tumor necrosis factor-α
Tregs:	regulatory T-cells
uPA:	urokinase plasminogen activator
VAP-1:	vascular adhesion protein-1
VCAM-1:	vascular adhesion molecule-1
VEGF:	vascular endothelial growth factor
VEGFR:	vascular endothelial growth factor receptor

INTRODUCTION

1. Metastasis

Metastasis is the process of cancer spreading to organs distant from the original tumor and constitutes a serious clinical problem. At the time of primary tumor diagnosis, metastasis has often already occurred and accounts for the death of the majority of cancer patients. Current therapeutic approaches directly target primary tumor progression whereas the prevention of metastasis provides a major challenge since our understanding of this complex process is still incomplete. Unravelling the intricate series of events during the metastatic cascade will therefore lead to new possibilities for therapeutic intervention.

1.1 The metastatic cascade: concepts and mechanisms

To successfully metastasize, tumor cells need to detach from the primary tumor mass and cross the endothelial lining to enter the blood stream. Tumor cells encounter many environmental challenges but can also cooperatively interact with host cells during intravascular transition. These interactions influence the metastatic potential of tumor cells and allow them to actively shape their environment, enabling them to exit the vascular system at secondary sites (Figure 1).

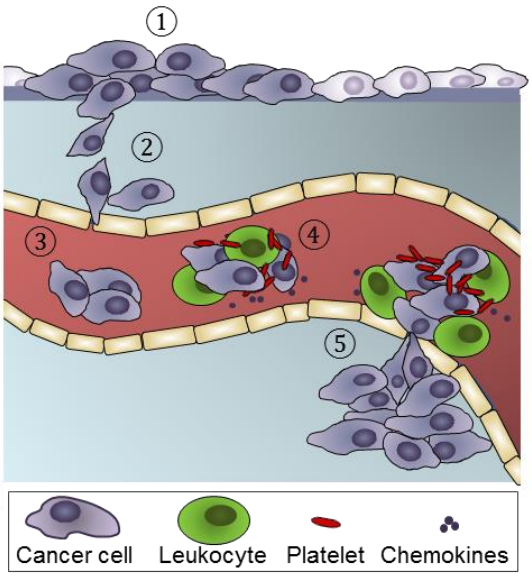

Figure 1. Metastatic cascade.
Hematogenous metastatic dissemination involves the following steps: ① Tumor cell detachment from the primary tumor site; ② Migration of tumor cells through the extracellular matrix; ③ Intravasation into blood vessels; ④ Tumor cell association with platelets and leukocytes; ⑤ Extravasation of tumor cell emboli and establishment of new metastatic lesions.

1.1.1 Concepts of metastasis

As discussed above, tumors can spread via the bloodstream which is known as hematogenous metastasis or via the lymphatic system to neighboring lymph nodes (lymphogenic metastasis). Since distant metastasis predominantly relies on hematogenous dissemination (1) this thesis focusses on metastasis via the blood stream.

Primary tumors constantly release millions of tumor cells into the circulation but nevertheless only very few metastases are established (2,3). In experimental animal models less than 0.01% of intravenously injected B16F1 melanoma cells were finally able to establish lung metastatic tumors (2,4). Later it was shown that most melanoma cells arrest in the microvasculature and extravasate into the liver parenchyma but fail to from persistent micrometastases in an experimental liver metastasis assay (5). Thus, metastasis is an inefficient process due to the fact that most tumor cells fail to execute successfully all steps of the metastatic cascade.

It has long been recognized that certain types of cancers specifically metastasize to distinct organs. For instance, breast cancer frequently spreads to bone, liver, brain and lungs whereas prostate cancers prefer to metastasize to bone. In turn, patients with colorectal cancer often show development of metastasis in the liver (3). This observation led to the formulation of the “seed and soil hypothesis” which explains that organ-specific dissemination patterns are a result of cancer cells (“the seed”) that are dependent on the environment (“the soil”) at the secondary organ (6). This view on metastasis was challenged by another hypothesis, which suggested that circulation pattern between a primary tumor and a secondary organ determine the organ specificity of metastasis (J. Ewing, Neoplastic Diseases, vol. 16, 6th edition, 1928). Both hypotheses are not mutually exclusive and current research supports a role for both concepts. Overall, circulating tumor cells will be preferentially taken to specific secondary sites depending on blood flow patterns but once tumor cells encounter a secondary organ, successful establishment of metastasis will depend on their compatibility with the organ’s environment (3,7).

An important parameter to predict the course of disease and therapeutically intervene with metastasis is the time point of cancer cell dissemination. Originally, metastasis was considered the final step in cancer progression. Current evidence from disease courses of different cancer types and genetics of disseminated tumor cells favors the parallel progression model (8) which proposes metastasis as an early event taking place before acquisition of fully malignant phenotypes (9-12).

1.1.2 Tumor cells in circulation

Cancer patients with solid tumors frequently have circulating tumor cells in their blood and a subset of these cells will eventually be able to establish metastatic tumors in distant organs (13,14). Most tumor cells get physically trapped in the microvasculature due to size restrictions within minutes after entering the circulation. However tumor cells in circulation and during initial arrest are also exposed to shear stress and to immune cells, especially natural killer (NK) cells so that not all arrested cells will form metastatic foci (3). During their short passage through the blood stream tumor cell properties enable them to escape the hostile environment in the blood vasculature. Tumor cell expressed tissue factor (TF) has been shown to trigger the formation of thrombin leading to coagulation as well as platelet activation, which has been associated with enhanced metastasis (15-19). The resulting fibrin binds to tumor cells as well as to activated platelets via integrins and thereby generates tumor cell-fibrin-platelet aggregates. Thus, activation of the coagulation cascade leads to platelet accumulation around tumor cells forming a protective barrier against shear stress and lysis by NK cells (15,20-23). Tumor cell interactions with platelets via P-selectin also significantly contribute to the formation of platelet-tumor cell thrombi (24,25) and will be discussed later. Many experimental models have identified platelets as crucial promoters of metastasis (26-30). Both platelet-derived TGF-β and platelet-derived growth factor (PDGF) have been shown to impair and suppress NK cell function (31,32). High platelet counts and coagulation are associated with poor clinical outcome (23,33,34).

Permanent adhesion to the endothelium is most probably mediated by specific interactions between tumor cells, immune cells and the vasculature. It is generally accepted that primary tumors are able to form so-called pre-metastatic niches which provide a supportive environment for incoming cancer cells (35-38). The primary tumor as well as tumor cell emboli themselves can activate the endothelium and platelets thereby mobilizing and recruiting different types of bone marrow derived cells (e.g. immature myeloid cells, neutrophils and monocytes) (38-43). The activated state of the endothelium may favor adhesion of tumor cells partially mediated by adhesion molecules such as selectins and other vascular adhesion molecules (25,44,45). Myeloid-derived cells in the metastatic microenvironment support tumor cells during the early stages of metastasis by promoting survival and facilitating extravasation of metastasizing cells (46).

1.1.3 Tumor cell extravasation

After intravascular arrest, tumor cells need to traverse the endothelium to successfully invade the parenchyma of a secondary organ. The observation that different tumor cells preferentially seed to different organs can be partially explained by the type of vasculature in the secondary organ. Bone marrow sinusoid capillaries for instance consist of fenestrated endothelia, normally facilitating leukocyte trafficking and are therefore permissive to circulating tumor cells (47). The fenestrated liver capillaries are also more likely to be crossed by tumor cells compared to other organs (48-50). On the contrary, pulmonary endothelial cells are surrounded by a basement membrane which impedes simple endothelial transmigration by tumor cells (51-54). Tumor cells produce specific mediators that enable them to bypass the capillary walls (55). For instance breast cancer cells have been shown to overexpress the adhesion molecule metadherin, which specifically binds to the vasculature in the lung and enhances metastasis (56). The concerted actions of chemokines and chemokine receptors expressed by tumor cells and the local environment contribute to tumor cell extravasation (57,58). Remarkably, all the initial interactions between

tumor cells and leukocytes, platelets or endothelial cells are primarily mediated by selectins which are considered to be major drivers of the metastatic process (25,59).

2. Selectins

Selectins are cell surface adhesion molecules that mediate the initial attachment of leukocytes to the endothelium during leukocyte extravasation at sites of inflammation and lymphocyte patrolling (60,61). Selectins are type-I transmembrane proteins consisting of an N-terminal calcium-dependent lectin domain, an epidermal growth factor-like (EGF) domain, two to nine consensus repeats, a single-pass transmembrane domain and a short cytosolic domain (62) (Figure 2).

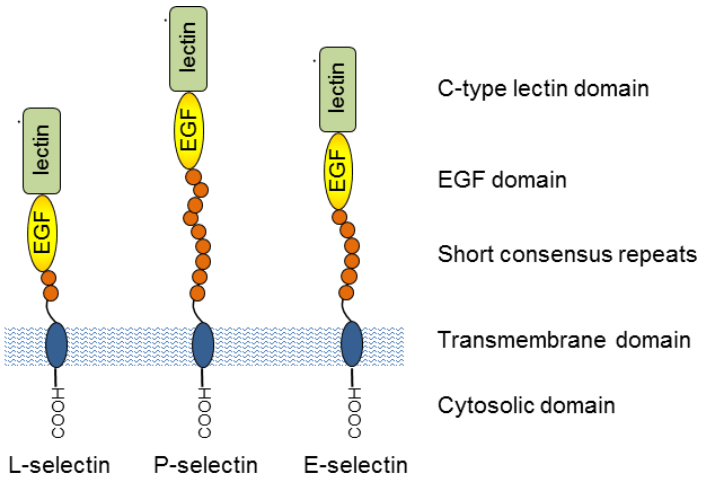

Figure 2. Structure of selectins. L-, P-, and E-selectin share a similar structure composed of a N-terminal calcium-dependent lectin domain, an epidermal growth factor-like (EGF) domain, 2, 9 or 6 consensus repeat units (L-, P- and E-selectin, respectively), and a single-pass transmembrane domain followed by a short cytosolic domain (62).

The selectin family contains three members that are L-, P-, and E-selectin which share around 50% sequence homology in their C-type lectin domain (60). Although the three selectins share many common elements they differ in the regulation of their expression, cell type specificity and binding specificities. L-selectin (LECAM-1, CD62L) is constitutively expressed by most hematopoietic cell types such as myeloid cells, lymphocytes, naïve T-cells or NK cells (60,63). L-selectin is best known as a mediator of leukocyte adhesion to the endothelium and to ligands expressed on high endothelial venules of the peripheral lymph nodes (64,65). P-selectin (PADGEM, CD62P) is stored in alpha-granules of unstimulated platelets as well as in Weibel-Pallade bodies of resting endothelial cells. Upon activation of

platelets or the endothelium P-selectin can be rapidly recruited to the cell surface. P-selectin plays an important role during platelet aggregation at sites of vascular injury while endothelial P-selectin contributes to leukocyte recruitment. E-selectin (ELAM-1, CD62E) is exclusively displayed by endothelial cells and expressed *de novo* in response to inflammatory stimuli such as TNF-α and IL-1β and mediates leukocyte rolling at sites of inflammation or injury. There is certain evidence for constitutive E-selectin expression in the skin and parts of the bone marrow (66).

2.1 Factors that regulate E-selectin expression

The expression of E-selectin is regulated by cytokines such as TNF-α and IL-1β which activate signaling pathways including JNK/p38 mitogen-activated protein kinase (p38 MAPK) and transcription factors like NF-κB and activator protein-1 (67-69). The amount of E-selectin on the cell surface peaks around 2 to 6 hours after stimulation and returns to its basal levels within 24 hours (70,71). In addition, other factors like shear stress (72), vascular endothelial growth factor (VEGF) (73), monocytes (74) and the activation of Rho family GTPases (75) trigger or prolong E-selectin expression. Cytokines such as TGF-β (76), glucocoriticoids (77) and the histamine H2-receptor antagonist cimetidine (78) suppress E-selectin expression.

2.2 Selectin ligands

The minimal carbohydrate motifs that are recognized by the lectin domain of all three selectins are the tetrasaccharide sialyl-Lewis^x and sialyl-Lewis^a (79) (Figure 3). Sialyl-Lewis^x (sLe^x; Neu5Ac α2,3 Galβ1,4 [Fuc α1,3] GlcNAc-R) is a terminal structure on N- or O-linked glycans attached to glycoproteins and glycolipids displayed by most circulating leukocytes and endothelial cells. Sialyl-Lewis^a however (sLe^a; Neu5Ac α2,3 Galβ1,3 [Fuc α1,4] GlcNAc-R) is found on limited types of epithelial cells but mostly on tumor cells (59,80). The four glycosyltransferases *N*-acetylglucosaminyltransferase, β1,4-galactosyltransferase, α2,3-sialyltransferase and α1,3-fucosyltransferase-4 or -7 sequentially synthesize sialyl-Lewis$^{a/x}$ carbohydrates in cells of the hematopoietic system (60) (59,61,81). Selectin ligands are often

located in clusters on glycoprotein scaffolds of the cell membrane which enables efficient binding of respective ligands (60). Modifications such as sulfation of the protein backbone or the carbohydrate recognition structure itself increase binding efficiency (82). Both P- and L-selectin can bind to sulfated glycans such as heparin, heparin sulfate, fucoidan and sulfated glycolipids (83,84).

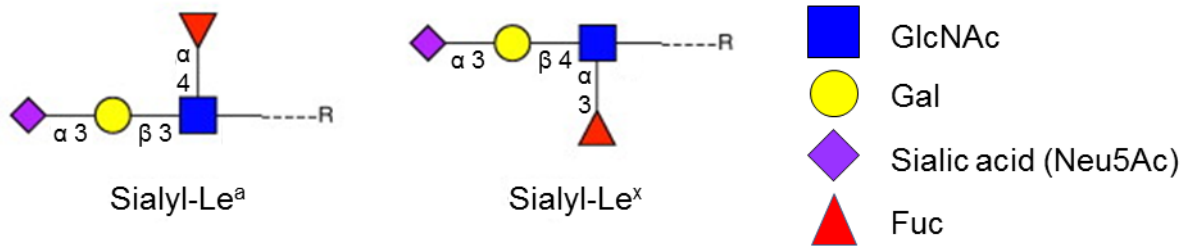

Figure 3. Structure of sialyl-Lewis^a and sialyl-Lewis^x (adapted from Juge *et al.*, 2012, Trends in Microbiology).

Neu5Ac α2,3 Galβ1,3 [Fuc α1,4] GlcNAc-R represents the structure of sialyl-Lewis^a whereas Neu5Ac α2,3 Galβ1,4 [Fuc α1,3] GlcNAc-R describes the structure of sialyl-Lewis^x.

To date P-selectin glycoprotein ligand-1 (PSGL-1) is one of the most thoroughly characterized ligands for all three selectins (65,85). PSGL-1 is concentrated on the tips of microvilli on the surface of leukocytes (86). Additional modifications of PSGL-1 core structures including sulfation of tyrosines Tyr48 and Tyr51 near the N-terminus of the protein potentiates binding of PSGL-1 to P- and L-selectin but not to E-selectin (87). PSGL-1 knock out mice have delayed neutrophil recruitment and moderate neutrophilia, similarly to P-selectin knock out mice (85,88). While P-selectin is the main receptor for PSGL-1, E-selectin also interacts with PSGL-1 besides other ligands such as E-selectin ligand-1 (ESL-1) and CD44. The interaction between E-selectin and PSGL-1 enables leukocyte capturing by the endothelium whereas the interactions of ESL-1 and CD44 with E-selectin mediate rolling of leukocytes (89). L-selectin binding to PSGL-1 expressed on leukocytes is thought to mediate collisional cell-cell interactions which initiate leukocyte aggregation thereby further recruiting inflammatory cells during pathological conditions (64). Other L-selectin ligands are primarily expressed on the luminal surface of high endothelial venules and play an important role for lymphocyte homing to secondary lymphatic tissues. These ligands comprise a

heterogeneous group of molecules called the peripheral lymph node addressin (PNAd) group which includes the glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), CD34, podocalyxin and endomucin. The mucosal addressin cell adhesion molecule-1 (MAdCAM-1) that is expressed in the gut on endothelial lymphatic tissue is also recognized by L-selectin (61).

2.3 Physiological roles of selectins

Selectins are critical regulators of leukocyte trafficking, like lymphocyte homing to secondary lymphoid tissues through high endothelial venules (90) or recruitment and extravasation of innate immune cells during pathophysiological conditions (91) (Figure 4).

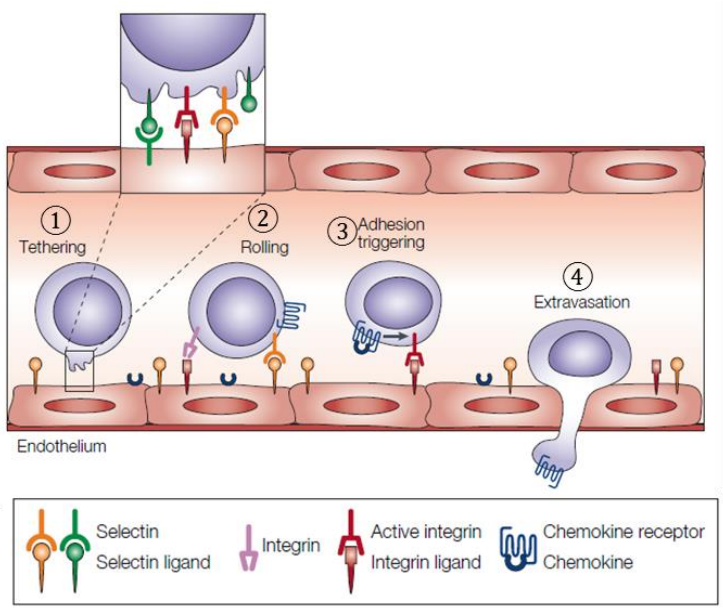

Figure 4. Leukocyte extravasation cascade.
Leukocyte extravasation is initiated by tethering ① of circulating leukocytes followed by rolling ② on the endothelium which is generally mediated by transient selectin–glycan ligand interactions and/or integrin interactions with vascular adhesion molecule-1 (VCAM-1) or intracellular adhesion molecule-1 (ICAM-1). During rolling, leukocytes are exposed to endothelial signals (such as chemokines) that trigger firm adhesion ③ to the endothelium by activating integrins on the leukocyte surface. Subsequently, leukocytes crawl along the vessel wall and ultimately migrate across the endothelium into the extravascular space ④ (adapted from Kunkel *et al.*, 2003, Nature Reviews Immunology).

During leukocyte recruitment and extravasation innate immune cells leave the intravascular compartment to enter sites of inflammation, infection or injury. This process involves several

tightly regulated steps such as selectin-mediated rolling on the endothelial cell layer, firm adhesion to the endothelia via integrins followed by leukocyte crawling and finally transmigration into the associated tissue (91-94) (Figure 4). During inflammation leukocyte recruitment mostly takes place in post capillary venules where hemodynamic shear forces are low.

2.3.1 The role of selectins during leukocyte rolling

The leukocyte adhesion cascade starts with freely circulating immune cells binding to the vessel wall which initiates their rolling along the endothelial lining. Rolling allows capturing of immune cells by the endothelium and is enabled by endothelial selectin binding to selectin ligands on leukocytes. During leukocyte rolling there is constant binding and release between selectins and their ligands due to their low affinity towards each other (95). Stagnating blood flow leads to the detachment of cells (95) while increased shear stress further strengthens binding (96). Studies with P-selectin deficient mice revealed a severe impairment of leukocyte rolling under inflammatory conditions (97). Although the speed of granulocyte rolling is increased in inflamed venules of E-selectin deficient mice, (98) the lack of E-selectin reveals no significant impairment of leukocyte recruitment in several models of inflammation (99). L-selectin is responsible for lymphocyte homing to peripheral lymph nodes (90) and upon binding to ligands extracellular domains of L-selectin can be rapidly shed from the leukocyte surface by proteolytic cleavage (100,101). L-selectin shedding possibly modulates the velocity of leukocyte rolling and regulates the degree of inflammation (102). The Inhibition of L-selectin shedding results in increased adhesion and transmigration of leukocytes due to prolonged leukocyte activation (103). Conversely, lymphocyte homing and leukocyte recruitment is affected in L-selectin deficient mice (104).

2.3.2 The role of integrins during leukocyte rolling

Next to selectins, integrins are another class of surface proteins expressed by leukocytes that can influence their rolling properties. Integrins are transmembrane receptors consisting

of α and β subunits and mediate cell-cell and cell-extracellular matrix (ECM) interactions. Integrins shift between different conformational states which determine their affinity towards the respective ligand. Furthermore, integrins are also capable of transducing signals across the cell membrane in both directions (105). α4β1 (VLA-4), α4β7 (LPAM), αLβ2 (LFA-1) and αMβ2 (Mac1, CD11b) are integrins which are expressed by leukocytes. α4β1 (VLA-4) binds to VCAM-1 and α4β7 interacts with VCAM-1 as well as with MAdCAM-1 and mediate leukocyte rolling independently of selectins (106-108).

2.3.3 Rolling-induced activation and signaling in leukocytes

The binding of selectins to their ligands induces intracellular signaling in the selectin- as well as selectin ligand-expressing cell (109-111). The signaling cascade which is induced in leukocytes by engaging with PSGL-1 is well studied and starts with signal transduction via immunoreceptor tyrosine-based activation motif- (ITAM-) containing adaptor molecules ezrin and moesin (110-112). These proteins link plasma membranes to actin cytoskeleton and are important for signaling during cell movement (113). Subsequently several non-receptor tyrosine kinases (e.g. Syk, Hek, Lyn, Btk) get activated and transmit signals to phospholipase C (PLC) and phosphatidylinositol 3 kinases (PI3K). This induces Calcium and DAG regulated guanine nucleotide exchange factor I (CalDAG-GEF-I) and MAP kinase p38 signaling pathways which activate Ras-related protein 1 (RAP 1). This leads to the recruitment of the FERM-containing protein talin to the cytoplasmic tail of integrins which mediates the changing of integrin conformation to an intermediate affinity state. As a consequence neutrophil rolling is slowed down (91,109,114-117).

2.3.4 Firm leukocyte arrest

In general selectin- or integrin-mediated rolling slows down leukocytes (95) and allows them to sense signals in the inflammatory environment. This enables chemokines, either bound to glycosaminoglycans such as heparan sulfate (118) on the endothelium or secreted by local macrophages, to activate G-protein coupled chemokine receptors (GPCR) on the leukocyte

cell surface (119). Actually, both selectin- and chemokine signaling pathways are required during leukocyte recruitment to fully activate integrins. These signaling events shift integrin conformation to an intermediate affinity state resulting in slower rolling of leukocytes (117). Consequently, chemokine signals accumulate thereby eliciting further conformational changes which finally leads to fully activated high affinity integrin conformations. This enables firm adhesion of leukocytes to their counter receptors presented on endothelial cells (119). Integrin and chemokine signaling during activation and firm adhesion of leukocytes also includes a distinct change in morphology of leukocytes. Thereby the round shape of rolling cells is transformed into a polarized morphology characterized by an F-actin rich front (lamellipodium) and a trailing edge (uropod) in which the cytoskeleton is contracted during migration. These changes allow the cells to coordinate intracellular forces required during crawling and subsequent transmigration (120-125).

2.3.5 Trans-endothelial migration

After selectin- and chemokine-induced, integrin-dependent leukocyte arrest, adhesion to endothelial cells is strengthened and lekocytes undergo cytoskeletal reorganization to spread and crawl along the vessel wall where they find a suited location for transmigration (also called diapedesis) through the endothelial lining (91). Diapedesis of leukocytes is either paracellular (through endothelial junctions) or transcellular (through the endothelial cell itself) (126,127). A recent study showed that approximately 90% of neutrophils exit the blood vessels via the paracellular pathway (128). The conditions which favor or determine the transmigration route of different leukocytes are currently not fully elucidated (129). Transmigrating cells interact with endothelial junctional molecules such as ICAM-1 which activates signaling pathways that elevate intracellular Ca^{2+} levels in endothelial cells (128-130). This is followed by the activation of myosin-light chain kinase causing cell contractions that open up adherens junctions between endothelial cells (130,131). Finally, VE-cadherin-β-catenin complexes that are indispensable for stabilization of endothelial junctions (129), dissociate what enables the opening of endothelial contacts. As a consequence, permeability

of endothelial cells is locally increased which enables leukocyte transmigration. Before reaching the interstitium, leukocytes need to pass the basement membrane that underlies the endothelial cell layer and the pericytes. Among others, integrin α6β1, PECAM-1 as well as basement membrane degrading proteases like the matrix metalloproteinases secreted by leukocytes help them to overcome the basement membrane (91,132-134).

2.3.6 Activation and signaling in endothelial cells upon E-selectin-ligand interaction

It has become apparent that engagement of E-selectin by its ligands on leukocytes also induces “outside-in” signaling in endothelial cells (135). After leukocyte binding to E-selectin or other events that mimic this process (e.g. antibody-mediated cross-linking or ligand-coated beads), a physical interaction of E-selectin with the actin cytoskeleton via its cytoplasmic domain is initiated and E-selectin clustering at the endothelial surface in the vicinity of adhering leukocytes is observed (136). E-selectin/ligand-interactions trigger the dephosphorylation of a usually phosphorylated serine residue in the cytoplasmatic domain of E-selectin (137). This signal seems to extend the half-life of E-selectin at the cell surface (138). At the same time a tyrosine residue (Tyr603) is phosphorylated, activating MAP kinase signaling pathways and consequently inducing transcriptional activation of the immediate early response gene c-*fos* (139,140). E-selectin has been demonstrated to localize into cholesterol-rich lipid rafts at the cell surface and upon ligation E-selectin is clustered and redistributed with a fraction of plasma membrane associated caveolin-1 containing rafts. After binding to a respective ligand, E-selectin that is localized in these lipid rafts associates with PLC-γ and drives its activation during leukocyte-endothelial interactions (135). There is also evidence indicating that the activation of E-selectin triggers cytoskeletal remodeling leading to the disruption of the VE-cadherin-β-catenin complex via the p38 MAP kinase signaling pathway and thereby regulating trans-endothelial permeability (51,141-143). Altogether, numerous *in vitro* studies show that E-selectin functions as an “outside-in” signaling receptor and highlight the importance of E-selectin as a regulator of endothelial barrier integrity.

2.4 Selectins in inflammatory diseases

Selectins are crucial for resolving infections and healing wounds however aberrant homing of leukocytes mediated by selectins has been associated with chronic or acute inflammatory pathologies, such as asthma (144-146), psoriasis (147-149), arthritis (150), acute ischemic stroke injuries (151) or with progression of cancer. E-selectin may also be involved in cardiovascular diseases since elevated levels have been found in hypertension, diabetes and hyperlipidemia (152).

2.5 Role of selectin-selectin ligand axis in tumor dissemination

All three selectins have been demonstrated to contribute towards tumor dissemination and is attributed to their ability to promote cell-cell interactions with tumor cells in their environment. Selectins specifically regulate adhesive interactions between circulating tumor cells and blood constituents such as platelets, leukocytes, and endothelial cells, thereby modulating the metastatic cascade at different steps (25,153-156).

During malignant transformation, physiological selectin ligands including PSGL-1, ESL-1, CD24, sLe^x, sLe^a, CD34, MAdCAM-1, lysosomal membrane glycoproteins LAMP-1 and LAMP-2, sulfatides, CD44 and death receptor 3 (DR3) that are normally presented by leukocytes can be up-regulated on the surface of tumor cells (157). Evidence obtained from clinical studies and *in vitro* data using human carcinoma cells as well as animal models with selectin deficiencies imply an important link between selectins and altered glycosylation on tumor cell ligands. It has been demonstrated that tumor cells which express sialylated or fucosylated molecules can be recognized by selectins (25,153,158-160) and that enhanced sLe^x and sLe^a expression correlates with poor prognosis due to enhanced tumor progression and metastatic spreading (161-163).

2.5.1 Carcinoma mucins

One example of an aberrantly expressed and glycosylated selectin ligand on carcinoma cells are mucin-type glycoproteins which bind to all three types of selectins. Mucins are high

molecular weight glycoproteins exhibiting a rod like conformation due to glycosylation with complex oligosaccharides, mainly constituting O-linked glycans (164,165). Mucin 1 (MUC1), MUC2, MUC4 and MUC16 are the most common mucins associated with cancer progression (165-168). Mucins exhibit glycan structures such as sLe^x, sLe^a, sialyted T and Tn antigens (169) which have long been associated with the progression of epithelial cancer and poor clinical prognosis of many human carcinomas such as colon cancer (170). By mediating adhesion to carcinoma mucins, platelet expressed P-selectin in combination with L-selectin is probably involved in triggering thromboembolic events in cancer patients described as Trousseau syndrome. This hypothesis is based on the observation that the injection of carcinoma cells into mice rapidly induced formation of platelet-rich microthrombi. Mice deficient in P- and L-selectin showed reduced microthrombi formation (171). Moreover, platelet-tumor aggregation and metastasis was attenuated by the enzymatic removal of selectin ligand carrying mucins from tumor cells before injecting them into the tail vein (172).

2.5.2 Sialyl-Lewis$^{x/a}$ during tumor progression and metastasis

Compelling clinical and experimental data demonstrates that overrepresentation of the tetrasaccharides sLe^x and sLe^a on tumor cell surfaces correlates with poor prognosis by potentiating metastatic behavior of various cancer types such as colon, gastric, prostate, renal, pancreatic and lung cancer (161,173-178). Human colon carcinoma cells with high sLe^x surface presentation showed more efficient colonization into the liver than cells expressing low levels of sLe^x in experimental metastasis models (179). sLe^a presence on the surface of colon cancer cells improved the growth of subcutaneous xenografts which was associated with enhanced angiogenesis (180).

Increased levels of $sLe^{x/a}$ on tumor cells have been attributed to the elevated expression of fucosyltransferase-7 which has also been shown to correspond with increased malignancy in lung cancer patients (174) and was further shown to be highly expressed in many colon cancer cell lines (181). The fucosyltransferases-3 and -6 have also been shown to be involved during sLe^x synthesis in multiple cancer cell lines and in colorectal cancer biopsies

(182-184). Studies investigating prostate and pancreatic cancer cell homing into bones revealed that E-selectin-mediated adhesion is dependent on elevated activity of the α1,3-fucosyltransferases-3, -6 or -7 (185,186). The genes encoding the sLe^x synthesizing fucosyltransferases-3 and -4 as well as ST3 β-galactoside α-2,3-sialyltransferase 6 (ST3GAL6), were found to be significantly overexpressed in breast cancers which correlated with metastasis to the bone where the sLe^x receptor E-selectin is constitutively expressed (187). Inflammatory cytokines such as TNF-α might also influence the production of sLe^x and contribute to metastasis by stimulating the expression of genes involved during the synthesis of selectin ligands (188).

2.5.3 P-selectin during the metastatic process

The association between circulating tumor cells and platelets, and the formation of tumor microemboli has been well established (30,189,190,191). Multiple studies have demonstrated that hematogenous dissemination, intravascular tumor cell survival and metastasis are enhanced by platelets (20,21,192,193). The adhesive interactions between platelets and tumor cells have been found to be primarily mediated by P-selectin. In P-selectin deficient mice, platelet-tumor cell interactions were significantly reduced and as a result these mice displayed attenuated metastasis (24). It was shown that platelet-association with tumor cells also prevented NK-mediated lysis of tumor cells (20). P-selectin accumulation around tumor cells is supposed to primarily occur during the initial phase of tissue colonization (194). In the last few years specific selectin ligands have been identified on tumor cells which interact with P-selectin. One example is the sLe^x motif carrying CD24, a mucin-type glycosylphosphatidylinositol (GPI)-linked cell surface glycoprotein that has been characterized as a P-selectin ligand (45,195,196) expressed by breast carcinoma cells. Chondroitin sulfate glycosaminoglycans on breast cancer cells function as P-selectin ligands and play an important role during breast cancer metastasis (197). Interestingly, it has been recently shown that pro-metastatic effects of platelets occurred earlier than the anti-metastatic effects of NK cells in murine lung metastasis models (193). This indicates other

mechanisms for the platelet-mediated facilitation of metastasis besides the concept of platelets shielding tumor cells form NK cell destruction. Next to platelet expressed P-selectin, endothelial P-selectin-mediated interactions were also shown to contribute to metastasis (193).

2.5.4 L-selectin during the metastatic process

L-selectin binds to a variety of tumor cells and participates during metastasis (153,198). In experimental metastasis assays, the injection of human and murine tumor cells into L-selectin deficient mice decreased the L-selectin mediated recruitment of leukocytes to tumor cell emboli and consequently attenuated metastasis. Synthesis of L-selectin ligands by fucosyltransferase-7 occurred around cancer cell emboli and correlated with the recruitment of leukocytes to intravascular tumor cell emboli (194,199). There is evidence that P- and L-selectin synergistically contribute to metastasis since metastasis has been attenuated in P- and L-selectin double deficient mice (194). These findings corroborate an active and dual role of L-selectin during metastasis which either facilitates leukocyte recruitment or interactions within the metastatic microenvironment. The enhanced infiltration of inflammatory cells, especially myeloid-derived cells is a hallmark of the tumor microenvironment and a well-characterized promoter of tumor growth and metastatic dissemination (39,41). L-selectin mediated recruitment of myeloid cells to metastatic sites may contribute towards the formation of the metastatic niche and promote early steps during metastasis, like tumor cell extravasation (43,199). Leukocyte interaction with the endothelium is known to induce vascular permeability during inflammatory processes. L-selectin presumably exerts is pro-metastatic effects by promoting an inflammatory microenvironment as well by directly interacting with tumor cells.

2.5.5 E-selectin during the metastatic process

The role of E-selectin during the metastatic process has been extensively studied during the past 30 years (156,157). Numerous clinical trials and plentiful experimental data led to the

hypothesis that E-selectin facilitates metastatic dissemination to distant organs by binding to ligands expressed on tumor cells comparable to leukocyte adhesion during inflammation (164).

In patients with metastasis high serum levels of E-selectin have been observed (200,201). This indicates that vascular E-selectin expression is increased in these patients as E-selectin is known to shed from the cell surface after expression and ligation (202). Increased E-selectin serum levels are associated with poor prognosis in patients with cancer cells expressing sLe^x (200,203,204). Numerous experiments *in vitro* applying physiological flow conditions provide evidence that tumor cells adhere to activated endothelium via their E-selectin ligands (155,205,206). As an example LS174T colon adenocarcinoma cells that possess sLe^x on glycoproteins and glycolipids were shown to adhere to HUVECS under physiological flow conditions *in vitro* (155). Human colon and hepatic carcinoma cells display sLe^x decorated core 2 branched O-linked carbohydrates which strongly bind to E-selectin and regulate invasiveness (184,205).

In vitro assays also revealed that cancer cell binding to endothelial E-selectin activates signaling pathways in endothelial cells which favor metastatic dissemination. E-selectin activation by colon cancer cells triggered the activation of both p38 and MAP kinases thereby inducing cytoskeletal remodeling (143,207). This generated breaches in the endothelial layer consequently facilitating extravasation of adhering cancer cells. The activation of E-selectin by binding its ligand disrupted the VE-cadherin-β-catenin complex in endothelial cells, which stabilizes cell-cell contacts, thereby contributing to E-selectin induced trans-endothelial permeability (143). These findings provide insights into mechanisms by which the adhesion of cancer cells to endothelial E-selectin may regulate the metastatic process.

2.5.5.1 E-selectin ligands involved in the metastatic process

Multiple E-selectin ligands that are expressed by cancer cells have been identified and linked to enhanced metastasis whereas most of them are mucin type molecules. Beside mucins, other E-selectin ligands presented on colon cancer cells have been characterized including

CD44, DR3, LAMP-1 and LAMP-2 (153,208-210). The CD44 transmembrane glycoprotein is involved during cell survival, cell adhesion, invasiveness, migration and angiogenesis (211) and is expressed by epithelial and endothelial cells as well as by multiple cancer cell types such as gastric, colorectal, pancreatic and lung cancer cells (212-214). Colorectal carcinoma cells that aberrantly express CD44 have an enhanced metastatic potential *in vivo* (215,216). Another E-selectin ligand is DR-3 which belongs to the TNF receptor family and was recently identified as a sialylated signaling ligand for E-selectin (208). Normally DR-3 is expressed by peripheral blood leukocytes and lymphocyte-rich tissue (217). DR-3 was also found to be expressed by metastatic colon cancer cells. Binding of DR-3 to endothelial E-selectin induced signaling via the MAP kinase p38 and thereby increased endothelial permeability and subsequently trans-endothelial migration of tumor cells (143,208).

The amount of E-selectin ligands is thought to determine the adhesive phenotype of cancer cells. Knocking down the often up-regulated fucosyltransferase-3 on circulating pancreatic cancer cells disrupted their adhesion to E-selectin *in vitro* (182). High mRNA levels of the enzyme core 2 β1,6-*N*-acetylglucosaminyltransferase (C2GnT1) which synthesizes the E-selectin ligand C2-O-sLe^x, have been found in colorectal adenocarcinomas (218). Such tumors were associated with vessel invasion, increased depth of tumor invasion and metastasis (219-221).

2.5.5.2 Experimental evidence *in vivo* for E-selectin as facilitator of metastasis

There is profound evidence that E-selectin promotes cancer metastasis in animal models. E-selectin expression was increased during metastatic liver colonization and down-regulation of E-selectin attenuated liver metastasis (143,222). In mice that overexpressed E-selectin in the liver, metastasis was redirected to the liver from the lungs in an experimental lung metastasis assay (223). This result provided direct evidence that E-selectin promotes tumor cell seeding. In line with this data, experimental liver metastasis of human colon carcinoma cells was also E-selectin-dependent (154). In contrast, metastasis of human colon adenocarcinoma was unchanged in an experimental lung metastasis model using

immunodeficient E-selectin knockout mice (224). Evidence from spontaneous metastasis models showed that primary tumors secrete factors which activate endothelial focal adhesion kinase and E-selectin in the lung vasculature and thereby induce the formation of hyperpermeable foci in lungs (225). This ultimately led to enhanced homing of cancer cells to these sites which correlated with metastasis. These findings imply that primary tumors are capable of actively priming and forming a distant metastatic niche by upregulating molecules involved in tumor cell-endothelial cell adhesion. Both E- and P-selectin have recently been shown to play a role during spontaneous metastasis formation into the bone marrow as well as into lungs in a xenograft model of human breast cancer (226).

Compelling clinical and experimental evidence support the concept of E-selectin as a facilitator of metastasis by enabling tumor cell adhesion to the vasculature. Nevertheless, many studies *in vivo* with E-selectin deficient mice do not provide exact mechanisms on how tumor cells interact with endothelial E-selectin and there is also controversial data about the organ and cancer cell type specific role of E-selectin during metastasis. Thus, details of the contribution of endothelial E-selectin to metastasis remain to be defined.

3. Primary tumor microenvironment

Cancer was previously considered a disease involving cells that had obtained oncogenic mutations which drive hyperproliferation and therefore tumor development and progression. During the past decades it has become evident that tumor cells do not cause cancer on their own. Bidirectional communication between tumor cells, normal tissue and bone marrow derived cells is essential to sustain tumor growth and progression. Tumor cells and stromal cells shape a tumor microenvironment (TME), which consists of resident structures such as endothelial cells and fibroblasts. These cell types provide a source of chemokines, cytokines and growth factors that mediate the recruitment of non-residential, infiltrating immune cells (227-229) (Figure 5).

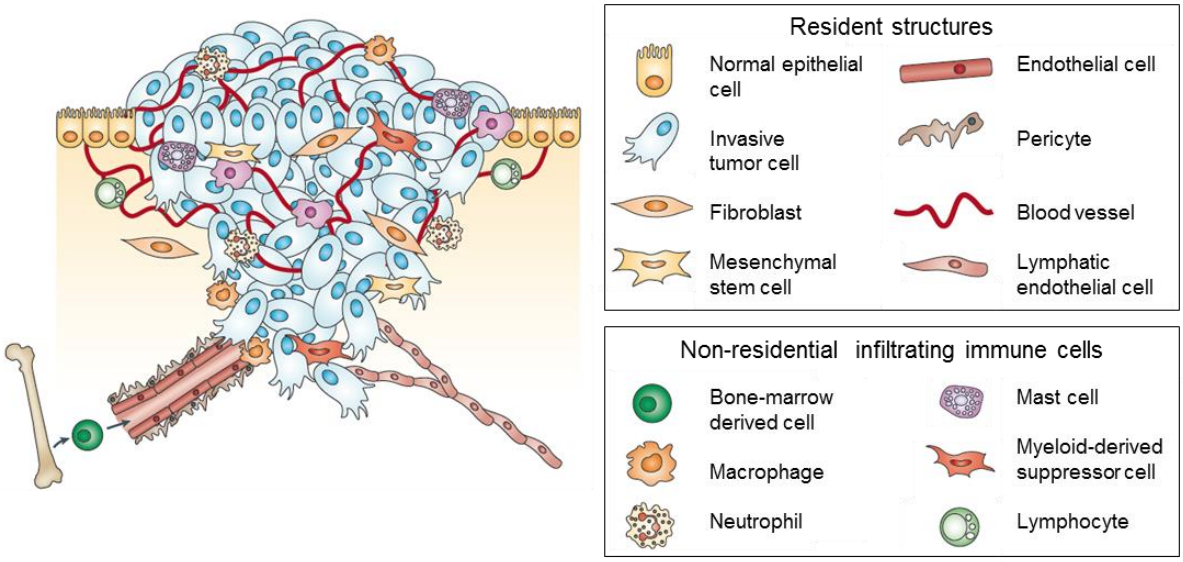

Figure 5. Components of a primary tumor.

A primary tumor mass consists of a complex microenvironment comprising various cell types which can be classified into resident and non-residential cells. Endothelial cells from the blood and lymphatic circulation, pericytes, stromal fibroblasts and mesenchymal stem cells are residential cells while bone-marrow derived cells such as macrophages, neutrophils, myeloid-derived suppressor cells, mast cells and lymphocytes are non-residential cells that infiltrate the primary tumor (adapted from Joyce *et al*., 2009, Nature Reviews Cancer).

3.1 Cancer-associated fibroblasts

Fibroblasts are part of the connective tissue, where they deposit ECM and basement membrane components. They can also modulate the differentiation of cells, immune responses and homeostasis (230,231). The tumor milieu contains high numbers of fibroblasts, so called cancer-associated fibroblasts (CAFs), which differ from normal fibroblasts. In breast cancer for example, CAFs can promote motile mesenchymal-like tumor cell morphology and thereby enhance the ability of malignant mammary epithelial cells to metastasize (232). In contrast normal fibroblasts favor an epithelial-like phenotype and suppress metastasis (232). The origin of CAFs during cancer development has not been completely elucidated (233). One hypothesis is that they arise from tumor-associated endothelial cells which detach from blood vessels to generate multipotent mesenchymal cells during epithelial-to-mesenchymal transition (EMT) (234). Several factors such as TGF-β, monocyte chemoattractant protein-1 (MCP-1/CCL2), platelet-derived growth factor (PDGF), fibroblast-growth factor (FGF) and secreted proteases can activate CAFs in the tumor

microenvironment (231,233). In turn, activation enables CAFs to supply the tumor milieu with secreted factors such as VEGF, which is known to induce vascular permeability and angiogenesis thereby supporting tumor progression (235).

3.2 Tumor vasculature

During angiogenesis neovascularization from pre-existing endothelial cells takes place, increasing the supply of the growing tumor with oxygen and nutrients. Angiogenesis has therefore been recognized as a key hallmark of cancer (236). Concerted interactions between cells from the TME, including vascular endothelial cells, pericytes and bone-marrow derived precursor cells enable tumor vascularization (237). Mesenchymal stem cells as well as CAFs are key players during tumor vascularization by secreting a variety of pro-angiogenic signals (238). Also cancer cells often express pro-angiogenic factors like VEGF that favor activation of angiogenic vessles (239). Endothelial cell-expressed factors including adhesion molecules (e.g. ICAM-1, VCAM-1, E- and P-selectin, hyaluronan) and chemokines such as IL-8, CCL2 and stromal-derived factor-1 (SDF-1) support the establishment of a vascular niche within the tumor milieu which promotes tumor progression and invasion (240,241). Endothelial cells have ECM remodeling capacities by secreting various proteases such as disintegrin, metalloproteinase domain containing protein 17 (ADAM17), matrix metalloproteinase (MMP) 2 or MMP10 and hence promote tumor growth (240). Tumor-associated blood vessels are not only involved during the promotion of tumor growth but also enable malignant cells to intravasate and disseminate (242). Activated endothelial cells also play an important role during the recruitment of immune cells to the neoplastic microenvironment where they regulate tumor angiogenesis (243).

3.3 Tumor-infiltrating immune cells

Inflammation and impaired immune responses have long been associated with tumorigenesis. Both are based on complex interactions between tumor and immune cells within the TME (244). Leukocyte infiltrates contribute a substantial fraction of the TME and

are centrally involved during neoplasm development. While some bone marrow-derived cells in the tumor milieu exhibit tumor-suppressive functions such as cytotoxic T-lymphocytes and NK cells, other types of leukocytes, especially innate immune cells including mast cells, immature myeloid cells, granulocytes and macrophages exert pro-tumorigenic effects. Upon entering the tumor mass, some leukocytes become alternatively activated and promote immune tolerance, tissue remodeling, cancer cell survival, angiogenesis, tumor growth and metastasis (245).

3.3.1 Tumor-associated macrophages

Tumor-associated macrophages (TAMs) are derived from peripheral reservoirs (e.g. bone marrow or spleen) or are residential in the tissue where they crucially regulate multiple aspects of tumor progression. Increased numbers of macrophages in a tumors stroma correlate with poor prognosis in many cancer diseases (246-249), has been linked to proliferation and survival of malignant cells, escape from immunosurveillance, angiogenesis, stroma remodeling, increased tumor size and metastasis formation (41,250-252).

Circulating macrophages primarily get recruited to tumors due to high concentration of tumor-derived chemoattractants such as the chemokines CCL2, CCL5, macrophage-colony stimulating factor (M-CSF/CSF-1), VEGF and PDGF (253-257). CCL2 for example is a well-recognized driver of macrophage infiltration in experimental and human tumors (258,259). CSF-1-mediated macrophage recruitment into neoplastic microenvironments is involved during malignant progression, angiogenesis and pulmonary metastasis in mouse models of aggressive metastatic mammary carcinogenesis (256,260).

Macrophages exhibit a plastic phenotype due to their capability to differentiate into various polarized cell stages depending on different environmental stimuli. This explains the disparate roles of macrophages during normal tissue homeostasis, infection and tumorigenesis. Classically activated M1 macrophages (i.e. by LPS/IFN-γ) are characterized by the production of pro-inflammatory cytokines including IL-1, IL-10, IL-23, TNF-α and CXCL-10 and can kill microorganisms as well as tumor cells via the Myd88/toll-like receptor

pathway (261,262). IL-4-, IL-10- and IL-13-exposed (263,264) monocytes on the other hand differentiate into M2 polarized macrophages and elicit anti-inflammatory responses which promote tumorigenesis (262,265). Environmental stimuli such as NF-κB-mediated inflammation as well as tumor hypoxia have been identified as regulators of TAM programming (266,267). Hypoxic regions in tumors have been shown to attract TAMs while also being associated with angiogenesis and an invasive phenotype (253). Upon arrival in the tumor microenvironment, TAMs produce factors such as EGF, members of the FGF family, TGF-β, VEGF, IL-6, IL-10, chemokines and proteases including urokinase-type plasminogen activator (uPA), MMP2 and 9 (268-271). These TAM-expressed proteins affect tumor proliferation and angiogenesis while driving dissolution of the connective tissues thereby favoring tumor cell invasion and migration (272,273).

3.3.2 Myeloid-derived suppressor cells

During pathological conditions such as sepis or cancer, myeloid cell differentiation is impaired, consequently giving rise to a heterogenous population of immature myeloid cells that exert immunosuppressive functions and are therefore termed myeloid-derived suppressor cells (MDSCs) (274). MDSCs are a mixture of monocyte- and granulocyte-like cells that are generated and released by the bone marrow after various stimuli (275). A common feature of this heterogeneous cell population is the repression of T-lymphocyte and NK cell effector functions (274,276). Therefore, tumor-infiltrating MDSCs are crucial regulators of cancer progression by compromising both innate and adaptive immunity by interfering with mechanism of immunosurveillance (277). Cancer patients have elevated numbers of MDSCs, which positively correlates with advanced disease and therapeutic inefficacy (278-280). In animal models, MDSCs have been shown to promote tumor progression as depletion with neutralizing antibodies significantly diminished metastasis (277,281,282). The janus tyrosine kinase and signal transducer and activator of transcription 3 (STAT3) is one of the most central transcription factors responsible for MDSC expansion in the tumor milieu (283). STAT3 up-regulation and reactive oxygen species (ROS) production

by MDSCs is induced by tumor cell-expressed factors such as VEGF and the inducible nitric oxide synthase (iNOS) and controlled MDSC recruitment and their immunosuppressive actions (284). STAT3 inhibition eliminated the suppressive activity of MDSCs in mice (285).

The immunosuppressive functions of MDSCs are attributed to T-cell suppression by cell-cell contact and the release of soluble mediators such as nitric oxide, ariginase-1, ROS or suppressive cytokines and chemokines (286,287). In B16F10 tumor bearing mice, MDSCs were shown to produce large amounts of the chemokine CCL5, which is required for melanoma cells and intratumoral regulatory T-cell (Tregs) expansion (288). Furthermore, MDSCs are capable of skewing the differentiation of $CD4^+$ T-cells to Tregs (289), polarizing macrophages to an M2 phenotype, impairing DC function and promoting angiogenesis (290).

3.3.3 Tumor-associated neutrophils

Several solid tumor types are infiltrated with neutrophils, including promyelocytes and mature granulocytes (291,292). Neutrophils are attracted to tumors and activated by diverse chemokines in the tumor microenvironment such as CXCL8 (IL-8), macrophage inflammatory protein-1 (MIP-1/CCL3) and granulocyte chemotactic protein-2 (GCP-2/CXCL6) (293-297). Similar to macrophages, neutrophils have a dual role during tumorigenesis. Transferring neutrophils into tumors has been shown to slow down tumor growth (298,299). But tumor-associated neutrophils (TANs) promoted malignancy by secreting growth stimulating signals, matrix-degrading proteases and angiogenic factors (300). These observations gave rise to the concept of anti-tumoral N1 and pro-tumoral N2 TANs which proposes neutrophil polarization and plasticity is regulated by the TME. TGF-β is thought to polarize TANs to a pro-tumoral N2 state and prevent the generation of a N1 stage. N1-like TANs express immunoactive cytokines and chemokines, have diminished arginase levels, exert enhanced abilities in killing tumors cells and activate cytotoxic T-lymphocytes (301). N2-like TANs on the other hand are a major source of CXCR4, VEGF and MMP-9 which support pro-tumoral functions and the production of SDF-1, IL-6 and CCL2 in the tumor milieu which further elevates neutrophil influx and survival (302). Oppositely, neutrophil-derived MMP-8 protects

against tumor development in animal models (303). Neutrophils also have cytotoxic and cytostatic functions by generating ROS upon activation which was shown to lyse various tumor cell types (304-308). However, extensive ROS action can be genotoxic and therefore result in pro-tumoral effects (309). Another way how N1-like TANs induce cell cytotoxicity is by influencing the activation state of $CD8^+$ T-cells by secreting cytokines, arginase and/or by activating dendritic cells (DCs) (301,310,311).

3.3.4 Lymphocytes in the tumor microenvironment

Tumor-infiltrating lymphocytes can favor the prognosis of tumors but some types of lymphocytes are also associated with pro-tumoral activities (312-315). One of the key players in cytotoxic anti-tumoral actions is the NK cell. In mouse models, NK cells promoted rejection of transplanted tumors, which was dependent on the presence or absence of NK cell receptor ligands on tumor cells. In contrast to other cytotoxic lymphocytes, NK cells are capable of killing cells by releasing perforin in the absence of tumoral MHC class I expression (316-320). NK cells play a fundamental role during tumor surveillance and further exhibit regulatory functions by interacting with DCs, macrophages, T-cells and endothelial cells (321-324). The role of NK cells in killing cancer cells was demonstrated in several melanoma mouse models, colon cancer, lung cancer and breast cancer (315,325-331).

Another class of lymphocytes recruited to the TME are cytotoxic $CD8^+$ T-cells which identify and kill cancer cells through tumor antigen recognition on MHC molecules (313). Similar to NK cells, $CD8^+$ T-cells produce perforin, granzyme B and high amounts of IFN-γ and have been shown to participate in tumor immunosurveillance in several animal cancer models (332-334). In melanoma patients, $CD8^+$ T-cells are present in high amounts in the circulation and found in metastatic lesions (335,336). Adoptively transferring cytotoxic T-lymphocytes into tumors is therapeutically effective in murine tumor models and is therefore emerging as a promising anti-cancer treatment (337).

A further substantial fraction of tumor-infiltrating lymphocytes are Tregs which can be observed in melanoma, lung and ovarian carcinomas, breast and colon cancers (338-342).

Due to their ability to inhibit effector functions of many immune cells such as T- and B-cells, DCs, macrophages and NK cells, Tregs are associated with suppressing anti-tumor activities and therefore favor the escape from immunosurveillance (343,344). However, Treg infiltration has also been associated with good prognosis in some types of cancers such as ovarian, bladder, head/neck and colorectal tumors (313,345). The mechanisms behind anti-tumoral actions of Tregs are still under investigation and are probably a consequence of the specific tumor milieu, which attracts different subtypes of Tregs (228,313).

$CD4^+$ T_{H1}- and T_{H2}-cells were also shown to play a role in the tumor microenvironment (346). While T_{H1}-cells can suppress tumor related inflammation, T_{H2}-cells exert tumor protective actions (347,348).

Although DCs do not belong to the lymphocytic lineage it's worth noting their important function as a link between the innate and adaptive immune system within the TME. Tumor-associated DCs possess the unique ability to present tumor-specific peptides to T-lymphocytes and thereby most probably elicit tumor-specific immune responses leading to rejection of tumors (336,349). On the contrary, the immunosuppressive tumor milieu can impair DC functions and consequently lead to failed T-cell activation (350,351).

3.3.5 E-selectin in primary tumors

The role of E-selectin in the primary tumor microenvironment is controversial and not completely understood. Reports claim that E-selectin is specifically up-regulated in melanoma by SDF-1α, which is thought to be critically involved in melanoma invasiveness (352). By down-regulating E-selectin, the homing of endothelial progenitor cells to the tumor and thus angiogenesis was markedly reduced, resulting in inhibited tumor growth of human melanoma xenografts in a SCID mouse model. Eliminating the E-selectin ligand-synthesizing α1,3-fucosyltransferases in a murine transgenic prostate adenoma model dramatically reduced cancer incidences (353). These findings suggest that E-selectin is engaged in pro-tumoral activities. However, selectins were also shown to contribute to tumor suppression by enabling the infiltration of anti-tumorigenic bone marrow-derived cells including macrophages

(354). The loss of E-selectin increased the growth of primary subcutaneous B16 melanoma cells, which was associated with reduced infiltration of NK cells, $CD4^+$ T-cells and $CD8^+$ T-cells (355). Clinical data demonstrate that high E-selectin expression correlates with improved survival in patients with Merkel cell carcinomas. In many Merkel cell carcinomas E-selectin expression is lost which is associated with poor intratumoral $CD8^+$ T-cell infiltration (356). This implies that E-selectin is the primary adhesion molecule for many types of tumor-suppressive immune cells. Vascular E-selectin expression in the tumor microenvironment may play a dual role and its effects on tumor progression might depend on different tumor cell types as well on the characteristics of the tumor milieu.

3.4 Metastasis initiating processes in the tumor microenvironment

In order to colonize distant organs tumor cells undergo phenotypic switching by acquiring specific features that enable them to free themselves from the primary tumor mass. The stromal compartment of a tumor contributes to tumor cell invasion and metastasis by enhancing invasive traits of tumor cells, enabling tumor cell detachment and by establishing a permissive environment at the target organ, the so called pre-metastatic niche (Figure 6).

Disaggregation of cancer cells from the tumor mass requires the loss of cell-cell adhesion within the primary tumor. Cadherin-catenin complexes maintain tight intercellular adhesive interactions (357). The loss of E-cadherin is a characteristic of invasive cancer cells and has been strongly linked with a metastatic phenotype (358-360). In numerous cancers including breast (361), gastrointestinal (362) and pancreatic cancers (363) a loss or down-regulation of E-cadherin has been observed. In a murine model of pancreatic cancer the disruption of E-cadherin resulted in early invasiveness and metastasis (358). To enter the blood stream, tumor cells must become motile and gain migratory traits. To achieve this they undergo an “epithelial-to-mesenchymal transition” (EMT) where they transform form a sedentary epithelial to a motile mesenchymal phenotype. EMT is mediated by molecules such as TGF-β, MAP kinases and the transcriptional regulators Twist, Snail, Wnt and Notch (364). Motile tumor cells are less sensitive to apoptosis (365) and more responsive to chemokine

gradients (366). Mesenchymal-type tumor cells are able to use proteases such as MMPs, cathepsins and uPA to degrade the ECM and the basement membrane (367-372). Once tumor cells have migrated though the stromal compartment and overcome the epithelial basement membrane and surrounding ECM, they reach tumor-associated vessels which are often not fully developed and therefore leaky (373) which facilitates cancer cell intravasation (374). Tumor vessel normalization that is characterized by increased vessel density, pericyte coverage and perfusion and decreased vessel size was reported to reduce metastasis (375). Stromal cell interactions with cancer cells play a fundamental role in supporting tumor cells to traverse structural boundaries of the primary tumor to reach the blood stream. TAMs produce factors such as FGF, EGF, PDGF, SDF-1 and proteases such as MMPs or cathepsins (376) which have been reported to enhance tumor cell migration and invasiveness (242,377,378). Mice with defective macrophages achieved by deleting the CSF-1 gene displayed impaired metastasis of breast cancer cells to the lungs (243,379). Other factors such as cytokines and growth factors that are released into the tumors stroma also provide pro-metastatic advantages. The cytokine TGF-β produced by stromal cells induces the expression of angiopoetin-like 4 that enhances metastatic activity and correlates with increased breast cancer metastasis to the lungs. Angiopoetin-like 4 has the ability to disrupt endothelial cell-cell junctions and thereby increases vessel permeability which enables tumor cells to intravasate as well as to exit the blood vessels at secondary sites (54). TGF-β is provided by TAMs, platelets and fibroblasts and is an important regulator of EMT in cancer cells (28,380-382). Altogether, metastatic dissemination is initiated by phenotypic switching of tumor cells which is potently driven by pro-migratory and ECM remodeling factors provided by stromal cells (4,383,384).

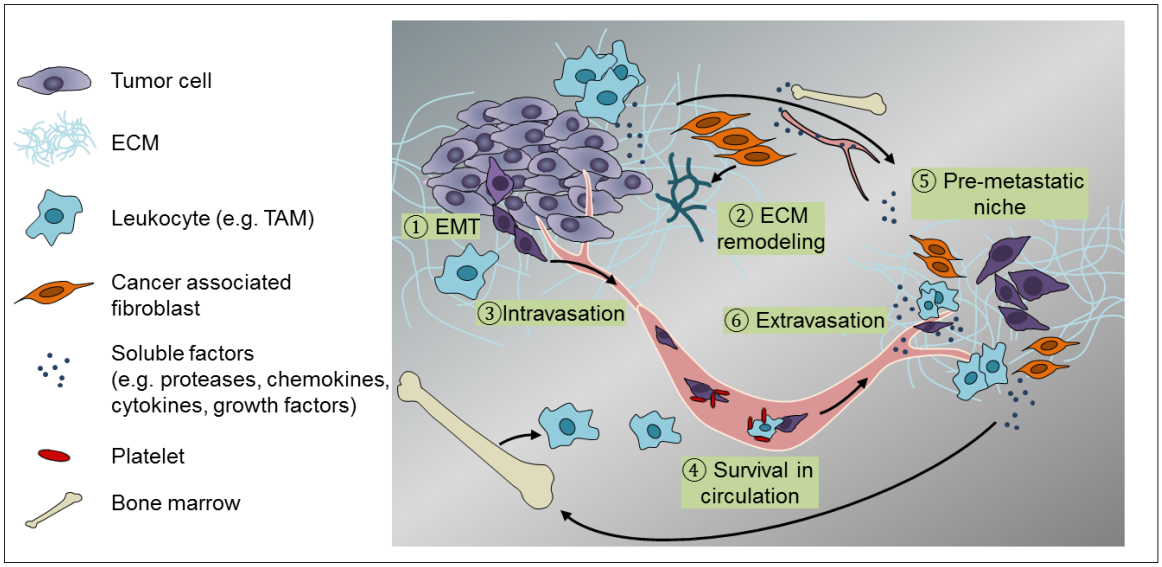

Fig. 6. From primary tumor to metastasis.

① Environmental stimuli such as hypoxia or cytokines induce EMT of tumor cells by activating various transcription factors leading to a motile tumor cell phenotype. ② Stromal immune cells including TAMs and CAFs strongly provide cancer cells with pro-tumorigenic proteases, cytokines and growth factors thereby supporting ECM remodeling and angiogenesis. ③ Tumor cells intravasate and form clots by associating with platelets and leukocytes. ④ This helps tumor cells to survive in circulation. ⑤ TME-derived factors prime future secondary sites by inducing clusters of bone marrow-derived cells. ⑥ This pre-metastatic niche facilitates tumor cell extravasation and engraftment (Based on Quail *et al*., 2013, Nature Medicine).

4. Metastatic microenvironment

Once a tumor cell reaches the circulation, intrinsic properties as well as exploiting host cells enable tumor cells to survive, firmly adhere to the vascular lining and eventually extravasate and colonize secondary sites. Beforehand, primary tumors elicit the release of signals such as cytokines and chemokines that direct host cells to distant organs. These host cells, especially bone marrow-derived myeloid cells, prepare a pre-metastatic niche providing a suitable and conductive environment for tumor cell engraftment (383,385).

4.1 Pre-metastatic niche formation

Several factors that are secreted by primary tumors can induce the clustering of specific cell populations such as hematopoietic progenitor cells and macrophages at future metastatic sites to create a permissive environment for tumor cell adhesion and extravasation (35,36). It

has been demonstrated that primary tumors upregulate fibronectin expression by residential fibroblasts at secondary organs, which attracts VEGF-receptor 1 positive ($VEGFR1^+$) hematopoietic progenitor cells that cluster at future metastatic sites (35). By depleting $VEGFR^+$ hematopoietic progenitor cells formation of pre-metastatic clusters can be decreased and metastasis blocked. $VCAM\text{-}1^+$ cancer cells interact with the VLA-4 integrin expressed by macrophages during breast cancer metastasis, leading to the protection of tumor cells from caspase-induced apoptosis (386). Metastasis to the bone is mediated by $VCAM\text{-}1^+$ tumor cell interactions with different integrin partners in osteoclasts (387). A hypoxic environment in breast cancer has been reported to induce the expression of lysyl odidase (LOX), which co-localizes with fibronectin at potential secondary sites and facilitates myeloid cell recruitment followed by tumor cell colonization in the lung. These myeloid cells are capable of degrading the basement membrane by MMP2 production and favored additional recruitment of myeloid cells (37,388). By inhibiting LOX in tumor cells myeloid cell attraction to the pre-metastatic niche was blocked and lung metastasis reduced (37). Other tumor-derived molecules that are involved while establishing a pre-metastatic niche include VEGF-A, TNF-α and TGF-β (36). These factors promote the expression of the inflammatory-related proteins S100A8 and S100A9 that consequently induce the expression of the chemoattractant serum amyloid A3 which stimulates NF-κB signaling via the toll-like receptor 4 (36,389). This pathway is responsible for the recruitment of both tumor cells and $CD11b^+$ myeloid cells to the pre-metastatic niche (36,389). S100A8 and S100A9 expression in cancer patients correlates with poor prognosis (390). The tumor-derived factor CCL5 has also been shown to promote the release of S100A4. Thereby pro-inflammatory cytokine-producing T-cells are attracted and subsequently leading to myeloid cell infiltration into the pre-metastatic environment (391,392). Hematogenic progenitor cells that accumulate at secondary sites revealed osteopontin expression, which has been associated with tumor cell adhesion and survival as well as with the regulation of MMP activity and host immune defense (393). G-CSF has been shown to mobilize $Ly6G^+Ly6C^+$ granulocytes to organ-specific metastatic sites which resulted in enhanced metastasis of several tumors (394). Recently, primary tumor-

derived exosomes were reported to shape their environment and generate a pro-tumoral niche, which attracts bone marrow derived progenitors that enhance metastatic dissemination (395,396). Exosomes are small biologically active vesicles that are derived from the late endosome and are involved in numerous extracellular activities such as cell signaling, immunological communication and cell recruitment (397). In conclusion, tumor cell specific traits and factors released by the TME mobilize and modify stromal cells, especially hematopoietic progenitor cells, to generate a fertile milieu at secondary organs which directs the establishment of secondary lesions (383,384,398).

4.2 Leukocytes, chemokines and selectins shape the metastatic environment

During the last 10 years research has generated knowledge about the concerted interplay between tumor cells, immune cells and chemottractants which support tumor cell adhesion and extravasation during metastasis. Selectins are considered to be adhesion molecules physically connecting the different players including tumor cells, endothelial cells, platelets and leukocytes. Therefore, selectins shape the metastatic environment and elicit further responses which facilitate tumor cell extravasation (Figure 7).

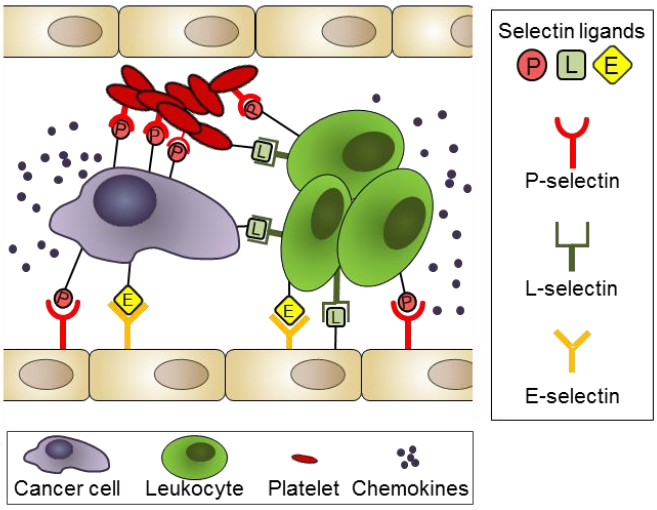

Figure 7. Selectins shape the metastatic environment.
P-, L-, and E-selectin mediate interactions between carcinoma cells, platelets, leukocytes and the activated endothelium. Thereby selectins modulate the capacity of cancer cells to adhere to and to transmigrate through the endothelium.

The activated endothelium is characterized by the increased expression of adhesion molecules and chemokines and is a well-recognized regulator of metastasis. Tumor cells that

enter the liver microvasculature induce TNF-α production by residential macrophages as well as vascular adhesion receptors such as E-selectin, VCAM-1 and PECAM-1 which promoted tumor cell arrest, extravasation and liver metastasis (399,400). Experimental metastasis models also showed that by reducing endothelial activation, the metastatic burden in animal models can be lowered (78,401). One assumption is that the presence of E- and P-selectin or other adhesion molecules on the activated endothelium is critically involved in mediating tumor cell survival, adhesion or recruitment of immune cells. P- and L-selectin-mediated tumor cell interactions with platelets and neutrophils in the microvasculature trigger the activation of the endothelium, which in turn produces the chemokine CCL5 (43). CCL5-mediated monocyte recruitment promotes tumor cell survival and metastasis (43).

The recruitment of myeloid cells, especially inflammatory monocytes, is strongly associated with enhanced metastatic colonization (40,43,199,402-404). The recruitment of $CD11b^+CD68^+F4/80^+$ macrophages by tumor cell clots mediated by tissue factor was shown to enhance the survival of arrested tumor cells during experimental lung metastasis (405). Tumor cell clot formation was also shown to induce the inflammatory marker VCAM-1 on the endothelium leading to myeloid cell recruitment, tumor cell survival and increased pulmonary metastasis (406). While there is compelling evidence for E-selectin as mediator of tumor cell adhesion to the microvasculature (see section 2.5.5) it remains to be elucidated whether E-selectin also participates in the interplay with other components of the metastatic environment.

Chemokines and chemokine receptors are strongly involved during the recruitment of immune cells to the metastatic environment and have been shown to support tumor cell extravasation. CCR1 was shown to control myeloid cell infiltration as well as angiogenesis thus promoting liver cancer metastasis in mice (407). $CCR1^+$ myeloid cells that have been recruited to the site of metastasis produce MMP2 and MMP9 and probably facilitate metastatic colonization (408). The CCL2/CCR2 signaling axis has been identified as a prominent contributor to tumor cell extravasation and metastatic colonization. Elevated levels of CCL2 are clearly associated with poor prognosis in breast, colon, prostate and cervix

cancer patients (409-412). The activation of the CCL2/CCR2 axis or CCL2 overexpression in prostate cancer cells promoted and increased metastasis to the bone, which was associated with enhanced macrophage recruitment (413,414). Similarly to these findings, CCL2-expressing breast cancer cells interact with $CCR2^+$ stromal cells of monocytic origin to favor colonization in lungs and bone (402). One mechanism which promotes CCL2 expression by tumor cells is mediated via NF-κB activation in tumor cells due direct contact with platelets leading to monocyte recruitment (28). Further evidence supporting the role of CCL2 as a facilitator of lung metastasis comes from a study which showed that $Gr1^+CCR2^+$ inflammatory monocytes are preferentially recruited to pulmonary metastases through CCL2, derived from both tumor and stromal cells (40,42). These studies also linked increased extravasation, seeding and growth of tumor cells via VEGF-dependent mechanisms to CCL2 signaling. CCL2 derived from colon carcinoma cells has been shown to directly signal to $CCR2^+$ endothelial cells which results in increased vascular permeability via the JAK2-Stat5 and p38 MAPK signaling pathways, consequently enabling tumor cell extravasation (403). A distinct population of CD11b/$Gr1^{(mid)}$ cells has been observed to strongly accumulate during the establishment of liver metastases due to tumor-derived CCL2 mediated recruitment from the bone marrow (415).

It is currently unknown whether the chemokine/chemokine receptor signaling axis directly impacts the expression of selectins or vice versa. CCL2/CCR2 signaling in endothelial cells engages the p38 MAPK signaling pathway (403), which is also involved in E- and P-selectin up-regulation during lung colonization (401). P- and L-selectin-mediated interactions led to the expression of CCL5 by endothelial cells (43) whereas the underlying mechanism is not defined. Nevertheless, this implies a link between the chemokine/chemokine receptor axis and selectin expression in the metastatic microenvironment.

The importance of selectin-ligand interactions within the metastatic environment has been recently highlighted by showing that selectin ligands on monocytes, especially PSGL-1, are crucial for monocyte recruitment to metastasizing tumor cells and enable efficient tumor cell survival, extravasation and metastasis (404). Thus, communication between tumor cells, the

endothelium and leukocytes are regulated by chemokines and mediated by selectins, which both substantially shape the metastatic niche. Our knowledge about this complex cooperation is incomplete and it requires profound research that will open new possibilities for therapeutic interventions.

4.3 Selectins and selectin ligands as therapeutic targets

Most anti-cancer therapies are designed to reduce the growth of primary tumors however therapeutic strategies, which aim to successfully prevent establishment of secondary lesions would provide a great alternative tool to improve the long term survival of cancer patients. It is important to emphasize that the time point of anti-metastatic drug administration might be crucial and often not optimal since metastatic events often precede the clinical diagnosis of malignancy. There have already been several attempts to interfere with the diverse selectin- and selectin ligand-mediated interactions during metastasis, but so far none of the potential candidates has been tested for clinical trials.

For the treatment of inflammatory diseases including psoriasis, graft-versus-host-disease, arthritis, asthma, atherosclerosis and ischemia-reperfusion injury, several anti-selectin therapies have been developed (416,417). Although experimental animal models have revealed promising effects, clinical trials have mostly been unsuccessful. One example is an engineered human anti-E-selectin antibody which showed protective effects in skin-versus graft disease in SCID mice by reducing leukocyte recruitment. However, the treatment failed to improve symptoms of psoriasis patients (418,419). By competitively inhibiting the interactions between selectins and their ligands with a recombinant soluble PSGL-1 immunoglobulin chimera (human PSGL-1 fused to a modified hinge region of human IgG1), leukocyte rolling and inflammation was prevented *in vivo* but this treatment was discontinued due to high production costs (416,417). Biamosiamose is a pan-selectin antagonist and revealed clinical improvement upon administration to psoriasis patients due to reduced leukocyte extravasation (420). Several other sLe^x mimetics such as cylexine or Efomycine M

have been tested in animal models of ischemia-reperfusion injury, psoriasis or asthma and have been evaluated to a certain extent in clinical trials (149,416,421,422).

Only a few studies have used such strategies to directly target selectins for anti-cancer treatments. In animal models anti-P-selectin monoclonal antibodies have been reported to suppress metastasis of gastric cancer with no adverse effects on immune functions (423). There have been attempts to inhibit the production of selectin ligands in cancer cells by using glycometabolic inhibitors which target the O-linked glycosylation of mucins or the activity of fucosyltransferases (421) shown to be involved during metastasis *in vivo* (424,425). Unfractionated and low molecular weight heparin exhibit metastasis suppressing effects in experimental mouse models probably by inhibiting P- and L-selectin-mediated interactions with tumor cells (426-428). Heparin is commonly used as an anticoagulant in the clinic and has been prescribed for treating cancer-associated thromboembolisms (Trousseau syndrome) (429,430). Its beneficial effects on the survival of patients (431) are most probably not only a consequence of anti-thrombotic activity but also due to selectins which are a major target of heparin (428).

Taken together, blocking selectins and their ligands to interrupt the metastatic cascade provides attractive prospects for treating cancer and thus, better knowledge of selectin-mediated communication within the metastatic microenvironment is essential.

5. Scientific aim

E-selectin is a well-studied facilitator of metastasis and historically considered to mediate intravascular tumor cell attachment. Interestingly, experimental data suggests that E-selectin is dispensable for tumor cell lodging in the microvasculature. In the present study we aimed to elucidate the role of E-selectin in metastasis by evaluating the interplay between E-selectin and the metastatic microenvironment during early metastatic phases. Identification of mechanisms how E-selectin interacts with different components of the metastatic niche may help to develop new approaches to therapeutically prevent metastasis.

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RESULTS

Manuscript: E-selectin-mediated monocyte adhesion and activation of the pulmonary endothelium induce vascular permeability and promote metastasis

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Manuscript

E-selectin-mediated monocyte adhesion and activation of the pulmonary endothelium induce vascular permeability and promote metastasis

Running Title: E-selectin-monocyte interaction promotes metastasis

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Key point 1: Tumor cell-induced endothelial activation induces E-selectin expression that promotes monocyte recruitment resulting in increased CCL2 levels during metastasis.

Key point 2: E-selectin-dependent monocyte interaction with the endothelium induces retraction of endothelial cells and thereby promotes tumor cell extravasation.

Abstract

During hematogenous metastasis tumor cells interact with blood constituents and these contacts enhance the capacity to colonize distant organs. Selectins are vascular cell adhesion receptors that contribute to the formation of a metastatic microenvironment but the role of endothelial E-selectin remains to be clarified. Here we show that E-selectin is a major adhesion receptor for the recruitment of monocytes to metastatic tumor cells. Experimental and spontaneous lung metastasis using murine tumor cells without E-selectin ligands (MC-38, B16-BL6 and LLC) was attenuated in E-selectin deficient mice. These findings provide evidence that E-selectin modulates metastasis also independently of a direct interaction with tumor cells. E-selectin-dependent myeloid cell recruitment further correlated with the accumulation of CCL2 chemokine in the metastatic lungs. E-selectin-mediated activation of both endothelial cells and monocytes contributed to enhanced CCL2 expression in the metastatic tissue. Monocytes supported tumor cell trans-endothelial migration which was dependent on E-selectin interaction with ligands on monocytes and resulted in an increased endothelial retraction. Accordingly, tumor cell-induced increase in lung vascular permeability was reduced in the absence of E-selectin. Thus, endothelial E-selectin expression during cancer progression shapes the tumor microenvironment through the recruitment, adhesion and activation of leukocytes that facilitate tumor cell extravasation and thereby metastasis.

Introduction

Hematogenous metastasis is a multistep process in which diverse interactions between tumor cells and their microenvironment allow the cells to cross physical boundaries, disseminate and colonize distant organs. Specifically, cell-cell interactions between tumor cells and blood constituents, such as platelets, leukocytes and endothelial cells, are initially mediated by selectins at different steps of the metastatic cascade (1-4).

Selectins are vascular cell adhesion receptors which are responsible for initial rolling and attachment of leukocytes to the endothelium and thereby enabling leukocyte trafficking and homeostasis (2,5). The selectin family consists of three members: E-, L-, and P-selectin which are present on activated endothelium, leukocytes or activated platelets and endothelium, respectively. Selectins recognize sialylated and fucosylated lactosamine terminal structures (called sialyl-Lewis antigens) displayed on leukocytes, platelets, and the endothelium or tumor cells. It is accepted that malignant transformation is associated with altered or excessive carbohydrate structure presentation on tumor cells, which correlates with poor prognosis due to metastasis (3,6).

E-selectin is the major leukocyte adhesion receptor that is present only on endothelial cells upon endothelial activation and requires *de novo* expression. E-selectin has been investigated as the primary receptor mediating tumor cell metastasis through facilitating adhesion of tumor cells on the endothelium (1). Several studies demonstrate that tumor cells expressing selectin ligands adhere to activated endothelium under flow conditions *in vitro* (7,8). There is also experimental evidence that E-selectin was up-regulated during metastatic liver colonization (9,10). E-selectin expression was observed in the microenvironment of tumor cells several hours after their lodging, indicating an inflammatory-like endothelial activation (11-13). Recently, E-selectin expression in the pre-metastatic lungs correlated with increased tumor cell homing to these tissues and with enhanced recruitment of myeloid cells (14). However, there is accumulating evidence that selectins contribute to stromal cell organization in the metastatic microenvironment (15,16). Accordingly, E-selectin is not required for tumor cell lodging in the microvasculature since endothelial E-selectin

expression is first induced after tumor cells are already present in the lungs (12). Thus the mechanism of E-selectin contribution to cancer progression requires further investigations *in vivo*.

Enhanced metastatic colonization of distant tissues is associated with increased recruitment of inflammatory, especially myeloid, cells (11,16-19). Among several chemokines potentially involved in metastasis, CCL2 appears to be the major chemokine involved in shaping up of the metastatic microenvironment primarily associated with the recruitment of monocytic cells. Interference in CCL2-CCR2 axis resulted in attenuation of metastasis of breast, lung, and colon tumor cells in various mouse models (18-21).

Considering the enhanced E-selectin expression upon tumor cell injection in variety of animal models, the role of E-selectin as a mediator of leukocyte recruitment can be expected. The presents study aims to elucidate the role of E-selectin in metastasis focusing on all participating cells which goes beyond a direct tumor cell binding to endothelial E-selectin.

Materials and Methods

Cell culture

Mouse colon carcinoma cell line MC-38 stably expressing GFP (MC-38GFP), Lewis lung carcinoma (LLC1; ATCC) and B16-BL6 melanoma cells were grown in DMEM medium with 10% FCS (19,22). Lewis lung carcinoma cells (3LL) were grown in RPMI medium with 10% FCS (12).

Mice

Animals were maintained under specific pathogen-free conditions, and experiments were according to the guidelines of the Swiss Animal Protection Law, and approved by Veterinary Office of Kanton Zurich. C57BL/6, Ccl2 deficient (*Ccl2*$^{-/-}$) and E-selectin deficient mice (*E-sel*$^{-/-}$) were purchased from The Jackson Laboratory. Fucosyltransferase 7 deficient mice (*Fuc-TVII*$^{-/-}$) were kindly provided by Dr. J.B. Lowe (University of Michigan, Ann Arbor, MI).

Experimental lung metastasis

MC-38GFP cells (300'000 cells) were intravenously injected (i.v.) into the tail vein of a mouse and metastatic foci were analyzed on day 28. 3LL and B16-BL6 cells (150'000 cells) were i.v. injected into mice and lungs were analyzed on day 14.

Spontaneous lung metastasis

LLC cells (200'000 cells) were subcutaneously injected into the right flank of mice and primary tumors were removed 18 days later when tumors reached a size of approx. 1 cm in diameter. Mice were terminated at day 30 and metastatic foci were counted on perfused lungs. Paraffin sections (5 µm) were stained with hematoxylin and eosin (Sigma-Aldrich), scanned with a Mirax Midi Slide Scanner (Zeiss) and analyzed with the Panoramic viewer 1.15.2 software (3DHISTECH).

Flow cytometry analysis

Mouse lungs perfused with PBS were minced and digested with Collagenase D and A (2 mg/mL each, Roche) for 1 hour at 37°C while shaking. Cells were separated using 18Gx1½ syringes and 40 µm cell strainers and erythrocytes were lysed using PharmLyse (BD Biosciences). Cells were incubated with anti-mouse CD16/32 mAb (eBioscience) and stained with fluorophore-conjugated antibodies against CD45, CD11b, F4/80, Ly6G, Ly6C and CD31 (BD Biosciences). Blood samples were treated with PharmLyse (BD Biosciences) and stained as described above. Data were acquired on a FACS Canto II (BD Biosciences) and analyzed using Flow Jo software (Tree Star).

Analysis of leukocytes, tumor cells and selectins in lung tissue

Frozen tissues were embedded in OCT (Sakura) as described previously (15). Cryosections (6 µm) were stained with following antibodies: CD11b; CD11b-biotin; Ly6G (BD Biosciences), F4/80 (AbD Serotec); and CD62E (BD Biosciences). Alexa568 conjugated anti-rat IgG or Alexa647-conjugated Streptavidin (Life Technologies) were used for detection using fluorescence microscope (Zeiss). Tumor cells and leukocytes were counted and the percentage of tumor cells associated with leukocytes was determined. The analysis of selectin and myeloid cell detection in lung sections was performed with a SP5 confocal microscope (Leica). Images were acquired of a total of 5 µm stacks and analyzed with Imaris Software (Bitplane).

Isolation of primary pulmonary endothelial cells

Pulmonary endothelial cells were isolated using a positive immuno-magnetic selection as described previously (19). Isolated endothelial cells were cultured in gelatin-coated 6-well plates in medium containing endothelial cell growth supplement (BD Biosciences).

Isolation of bone marrow monocytes

Femur, tibia and pelvic bones were harvested from mice and crushed in PBS containing 2% FCS and 2.5mM EDTA. Red blood cells were lysed using ammonium chloride solution. Bone marrow derived monocytes were enriched by magnetic activated cell sorting (MACS) using

biotinylated macrophage colony-stimulating factor receptor (M-CSFR, CD115) antibody (Biolegend) and streptavidin-conjugated magnetic beads (Miltenyi Biotec).

Monocyte recruitment in a microfluidic channel system

Primary lung microvascular cells (175'000 cells) were plated on gelatin-coated µ-Slide $I^{0.2}$ Luer (ibidi GmbH) and were allowed to grow to confluence for 2 days. The endothelial monolayer was manually perfused five times with 200'000 MC-38GFP cells in 100µl media. After 4 hours slides were perfused with CellTrace calcein red-orange, AM (Life Technologies) stained bone marrow monocytes ($2x10^6$ cells/ml) in HEPES buffered Ringer solution supplemented with 25% washed red blood cells. Perfusion of slides was performed using an ibidi air pressure pump system (ibidi) applying a flow rate of 2 dyne/cm^2. Mosaic images of slides were acquired using an inverted fluorescence microscope (Zeiss Axio Observer Z.1) at indicated time points. Pictures were analyzed with ZEN software (Zeiss).

Vascular permeability assay

Vascular permeability in the lungs was determined with Evans blue dye extravasation technique (19). Briefly, 24 hours after mice were injected with tumor cells 2 mg of Evans blue was injected i.v. followed by euthanasia 30 min later. Extracted Evans blue was measured using spectrophotometer (620 nm). Clodronate liposomes (1.8 mg) were i.v. injected to deplete monocytes (23) 24 hours prior to tumor cell i.v. injection, followed by Evans blue injection 24 hours later.

RNA isolation and quantitative real-time PCR

Perfused and snap frozen lungs of mice were used for a total RNA isolation using TRI Reagent (Sigma-Aldrich) according to the manufacturer's protocol. The quantity and quality of the RNA was determined spectroscopically using a Nanodrop (Thermo Scientific). Isolated RNA was reversely transcribed into cDNA using Omniscript RT Kit (Qiagen) according to the manufacturer's protocol. Real-time PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in combination with the MX300P light cycler (Agilent). Data were

analyzed using MxPro Mx300P software (Agilent) and normalized to the housekeeping gene *Gapdh*. Primers separated by an intron were used (Table S1).

RNA isolation from sorted pulmonary monocytes and endothelial cells

PBS-perfused lungs were minced and digested in Collagenase D and A (2 mg/mL each, Roche) for 1 hour at 37°C. A single-cell suspension was prepared by passing the digested lungs through 18Gx1½ syringes, 100µm and 40µm cell strainers (BD Biosciences). Cells were incubated with rat antibodies against CD31, CD45, CD11b, Ly6C and Ly6G (eBiosciences). Endothelial cells were sorted as $CD45^{-}CD11b^{-}CD31^{+}$ cells and inflammatory monocytes were sorted as $CD45^{+}CD11b^{+}Ly6C^{high}Ly6G^{-}$ cells with a FACSAria III sorter (BD Biosciences). Total RNA of sorted cells was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Real-time PCR was performed as described above.

Cytometric bead array

Perfused lungs of mice were homogenized, supernatant was collected and concentration of the chemokine CCL2 was assessed by using the cytometric bead array Kit for mouse CCL2 (BD Biosciences) according to the manufacturer's protocol. CCL2 levels were normalized to the total protein amount of lung supernatants.

Trans-endothelial migration assay

Primary lung endothelial cells (25'000) were seeded on gelatin coated 24-well transwell inserts (8µm pores; BD Biosciences) and allowed to grow to confluence for 2 days. MC-38GFP cells (25'000) were seeded into transwell inserts with or without monocytes (200'000) purified from bone marrow (untreated or fixed) in 3% FCS/RPMI in the upper chamber and 10% FCS/RPMI was added into the lower chamber. When fixed monocytes were used, cells were fixed with 2% PFA for 12 minutes at 4°C and washed prior to use. After 16 hours of co-culture, the upper side of the insert was scraped off, the insert was fixed in 2% PFA and nuclei were stained with DAPI. Tumor cells on the lower side of the membrane were analyzed with a fluorescence microscope (Zeiss).

Staining of endothelial cytoskeleton

Endothelial F-actin was detected with Phalloidin staining (24). Briefly, 40’000 pulmonary endothelial cells grown on gelatin-coated chamber slides for 36 hours were co-incubated with MC-38GFP cells (40’000) stained with a PKH26 red fluorescent dye (Sigma-Aldrich) and bone marrow monocytes (200’000 cells) for 8 hours. Cells were fixed with 2% PFA, permeabilized (0.1% saponin), and stained with Phalloidin-FITC (6.67 mg/ml, Sigma-Aldrich). Nuclei were stained with DAPI and cells were mounted in ProLong Gold (Life technologies).

Statistical analysis

Statistical analysis was performed with the GraphPad Prism software (version 5.01). All data are presented as mean ±SEM and were analyzed by ANOVA with the post-hoc Bonferroni multiple comparison test. Analysis of two samples was performed with Mann-Whitney test unless stated otherwise.

Results

E-selectin facilitates experimental metastasis of tumor cells with no E-selectin ligands

During cancer progression it has been postulated that E-selectin binding to tumor cells facilitates tumor cell lodging in the microvasculature and thereby metastasis (1,25). To assess whether a direct interaction of tumor cells with E-selectin is required for metastasis, we tested mouse adenocarcinoma cells (MC-38GFP) in an experimental metastasis model. MC-38GFP cells were confirmed to express no E-selectin ligands while P- and L-selectin ligands were present (Figure S1A). Intravenous injection (i.v.) of MC-38GFP into C57BL/6 (wt) and E-selectin deficient (*E-sel*$^{-/-}$) mice revealed a significant reduction in the number of pulmonary metastatic foci and total tumor burden in *E-sel*$^{-/-}$ mice compared to C57BL/6 mice (Figure 1A-B, S1B). To confirm that this finding is not cell line specific we used Lewis lung carcinoma cells (3LL) and melanoma cells (B16-BL6) in the same experimental model (Figure 1C-F). Both 3LL and B16-BL6 cells do not express any E-selectin ligands (Figure S1A). We observed reduced metastasis in *E-sel*$^{-/-}$ mice with both cell lines (Figure 1C-F). Since E-selectin is expressed only upon endothelial activation and the fact that in experimental metastasis model tumor cells are present in the lungs prior to this point (12), these data strongly indicate that E-selectin promotes metastasis without directly interacting with tumor cells but through interaction with other cells within the tumor microenvironment.

E-selectin-dependent leukocyte infiltration of the metastatic tissue - lungs

Leukocytes constitute a crucial part of the metastatic microenvironment supporting tumor cell extravasation (16-19). To assess whether E-selectin facilitates metastasis through recruitment of leukocytes, we analyzed the early metastatic microenvironment in the lungs of mice. Tumor cell-injected mice were terminated 24 and 48 hours later and perfused lungs were analyzed by flow cytometry. Total leukocyte infiltration was significantly increased in C57BL/6 mice 48 h post-tumor cell injection (p.i.) but remained unchanged in *E-sel*$^{-/-}$ mice (Figure 2A). Further analysis revealed significant increase in the number of inflammatory monocytes (Ly6C^{high}) and macrophages (F4/80^{+}) in C57BL/6 mice compared to *E-sel*$^{-/-}$ mice

(Figure 2B-C). However, no difference was found in granulocyte ($Ly6G^+$) infiltration to lungs of mice at these time points (data not shown). We further analyzed the number of leukocytes and tumor cells on lung cryosections. Reduced numbers of macrophages ($F4/80^+$) were found at 16 and 24 hours p.i. in *E-sel*$^{-/-}$ mice compared to C57BL/6 mice (Figure 2D and F). We observed more granulocytes ($Ly6G^+$) only at 16 hours p.i. in C57BL/6 compared to *E-sel*$^{-/-}$ mice (Figure 2E and F). The parallel analysis of peripheral blood cells from naïve mice revealed no difference in numbers of $CD11b^+$, $F4/80^+$, and $Ly6G^+$ cells between *E-sel*$^{-/-}$ and C57BL/6 mice (Figure S2D). Thus, the missing monocyte infiltration in *E-sel*$^{-/-}$ mice indicates that E-selectin expression is critical for the leukocyte recruitment to the metastatic organ/lungs.

To assess the role of E-selectin-dependent leukocyte recruitment on tumor cell survival, we quantified the number of tumor cells in the lungs of mice 16 and 24 hours p.i. Significantly decreased number of viable (GFP-positive) tumor cells in the lungs of *E-sel*$^{-/-}$ mice was observed at both time points indicating an impairment of early metastatic events (Figure 2G).

Tumor cell-induced E-selectin expression facilitates leukocyte recruitment to metastatic cells

The observed difference in leukocyte infiltration to the lungs indicates that E-selectin is present during early phase of metastasis. We analyzed E-selectin expression in the lungs upon tumor cell injection at different time points by real-time PCR. Maximal E-selectin expression was observed 6 hours p.i. which was reduced almost to baseline levels 12 hours p.i. (Figure 3A). Next we detected E-selectin in the vicinity of tumor cells 6 and 14 hours p.i. in C57BL/6 mice using immunofluorescence microscopy (Figure 3B). Importantly, myeloid cells ($CD11b^+$) were detected in the areas positive for E-selectin, which was always in the vicinity of tumor cells (Figure 3C). To assess whether E-selectin facilitates leukocyte recruitment to metastatic tumor cells we analyzed the interaction between tumor cells and leukocytes on lung sections. A significant reduction in both $F4/80^+$ cell and $Ly6G^+$ cell interactions was detected at 16 hours p.i. in *E-sel*$^{-/-}$ mice compared to C57BL/6 mice (Figure 3D). However, only $F4/80^+$ cell interactions remained significantly different also 24 hours p.i.

To test whether E-selectin is indeed involved in leukocyte recruitment to metastasizing tumor cells in the microvasculature we analyzed this process using a microfluidic system *in vitro* (Figure S3). We assessed monocyte recruitment to MC-38GFP cells adherent on primary lung endothelial monolayers derived from C57BL/6 and *E-sel*$^{-/-}$ mice under physiological post-capillary flow conditions. Reduced number of monocytes was bound to endothelial cells around attached tumor cells on *E-sel*$^{-/-}$ derived endothelial monolayers compared to C57BL/6-derived endothelial monolayers (Figure 3E-F). We observed reduced monocyte adhesion already at 15 min after initiation of the perfusion and this effect lasted for at least 90 minutes. In addition, the number of monocytes present within the vicinity of a tumor cell was also significantly reduced on endothelial monolayers derived from *E-sel*$^{-/-}$ mice (Figure 3G). Given the absence of E-selectin ligands and therefore the inability of MC-38GFP cells to interact with the E-selectin, these *in vivo* and *in vitro* data provide evidence that E-selectin facilitates monocyte recruitment to metastasizing tumor cells.

Intravascular tumor cells induce endothelial activation and E-selectin contributes to an increased CCL2 pool in metastatic lungs

E-selectin expression is an established marker of endothelial activation that is observed both in inflammatory and cancer-related situations (2,5). Next, we analyzed the activation status of the lung endothelium in response to tumor cell injection. Expression levels of vascular adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) were increased upon tumor cell injection in lungs of C57BL/6 mice but remained unchanged in lungs of *E-sel*$^{-/-}$ mice 6 and 12 hours p.i. compared to naïve mice (Figure 4A-B). In addition, we observed significant increase in expression of chemokine CCL2 and its receptor CCR2 in the lungs of C57BL/6 mice 6, 12 and 24 hours p.i., while only a minimal increase in CCL2, but not CCR2 in *E-sel*$^{-/-}$ mice could be detected (Figure 4C and D). CCL2 is produced both by MC-38GFP cells and by stromal cells and the CCL2/CCR2 signaling axis has been associated with recruitment of inflammatory monocytes, vascular permeability and tumor cell extravasation (17-19). The reduced CCL2 expression levels in *E-sel*$^{-/-}$ mice 12 and 24 hours p.i. can be explained by the reduced numbers of leukocytes or tumor cells in the

microvasculature of the lungs (Figure 2D-G). However, comparable number of tumor cells and leukocytes are present in C57BL/6 and *E-sel*$^{-/-}$ mice 6 hours p.i., respectively (data not shown). This observation indicates that high CCL2 expression results from endogenous upregulation by endothelial or myeloid cells, that is dependent on E-selectin.

Next we assessed the role of CCL2 from tumor cells on the metastatic microenvironment by injection of MC-38GFP cells with a silenced CCL2 expression by 50% (MC-38GFP CCL2 KD) and compared with wild type MC-38GFP cells 6 hours p.i. (Figure S4A). We observed significantly reduced endothelial activation in mice injected with MC-38GFP CCL2 KD as measured by E-selectin and VCAM-1 expression; and similarly reduced CCL2 and CCR2 expression (Figure 4E-G and S4B). These results indicate that the reduced CCL2 expression by tumor cells resulted in impaired endothelial activation indicating a possible direct involvement of tumor cell-derived CCL2 in endothelial activation and thereby the expression of E-selectin required for leukocyte recruitment.

To test how tumor cell injection changes the CCL2 levels within the metastatic lungs, we analyzed CCL2 protein concentrations in C57BL/6, *E-sel*$^{-/-}$, and *Ccl2*-deficient mice (*Ccl2*$^{-/-}$) 6 and 12 hours p.i. Compared to untreated mice (3 pg) CCL2 protein levels were significantly elevated in C57BL/6 mice (27 pg) while a diminished increased in lungs of *E-sel*$^{-/-}$ mice (11 pg) was detected 6 hours p.i. (Figure 4H). In lungs of *Ccl2*$^{-/-}$mice we observed minimal amounts of CCL2 (2 pg), which reflected the tumor-derived CCL2. The concentration of CCL2 remained high in the lungs of C57BL/6 mice also 12 hours p.i., albeit slightly decreased (Figure 4I). Thus, the strong increase in CCL2 in tumor cell-injected C57BL/6 mice showed that the majority of CCL2 is derived from the local microenvironment. Furthermore, the significant reduction of CCL2 pool in *E-sel*$^{-/-}$ mice strongly argues for the E-selectin involvement in chemokine production. To address the source of CCL2 production in metastatic tissue, we sorted endothelial cells (CD31^{+}) and myeloid cells (CD11b^{+}/Ly6C^{high}) from lungs of mice 12 hours p.i. and analyzed CCL2 expression. We observed significant increase in CCL2 expression in endothelial cells from C57BL/6 compared to naïve mice or *E-sel*$^{-/-}$ mice (Figure 4J). Interestingly, CCL2 expression was also increased in sorted

monocytes only from C57BL/6 mice, indicating that E-selectin-mediated activation of monocytes induces CCL2 expression. Indeed, the absolute CCL2 expression levels were mostly derived from monocytes (Figure S4C). These data provided evidence that CCL2 expression is induced by E-selectin-mediated activation of both endothelial cells and monocytes, which together contribute to the increased chemokine pool observed in the metastatic lungs.

E-selectin-dependent activation of endothelial cells through selectin ligands on monocytes promotes tumor cell trans-endothelial migration

Increased levels of CCL2 correlate with metastatic progression in various mouse models, and contribute to tumor cell extravasation (18-20,26). To test whether E-selectin expression is required for tumor cell extravasation, we analyzed lung vascular permeability 24 hours p.i. using the Evans blue assay (19). Tumor cell injection induced lung vascular permeability in C57BL/6 mice but was almost entirely absent in *E-sel*$^{-/-}$ mice (Figure 5A-B). Next we tested the hypothesis that the E-selectin-mediated recruitment and activation of monocytes contribute to induction of vascular permeability. We used clodronate liposome injection protocol to deplete peripheral circulating monocytes (F4/80^{+}), followed 24 hours later by intravenous injection of MC-38GFP cells. Evans blue assay was performed 24 hours p.i. Monocyte-depleted C57BL/6 mice showed significantly decreased vascular permeability compared to untreated mice (Figure 5C-D). These data provided evidence that monocyte recruitment facilitates tumor cell extravasation by E-selectin-mediated leukocyte-dependent endothelial activation leading to increased vascular permeability.

To further explore the role of E-selectin by tumor cell extravasation, we studied the capacity of tumor cells to transmigrate through endothelial cells in presence of monocytes (Figure S5). We used lung microvascular endothelial cell monolayers derived from C57BL/6 and *E-sel*$^{-/-}$ mice and studied the transmigration of MC-38GFP cells in the presence of monocytes (CD115^{+}) (Figure 5E-F). While tumor cells have an intrinsic ability to migrate through endothelial cells, the presence of monocytes significantly promoted this process (19). However, there was no increase in tumor cell transmigration through E-selectin deficient

endothelial cells in presence of monocytes (Figure 5E-F). This observation suggests that E-selectin binding to monocytes is essential for trans-endothelial migration. To test this hypothesis, we used fixed monocytes, which only present ligands on their surfaces, in the transmigration assay using endothelial cells from C57BL/6 mice (Figure 5G). Notably, fixed monocytes increased the tumor cell transmigration albeit not to the same level as unfixed cells. Next we used monocytes derived from fucosyltransferase-7 deficient mice (*Fuc-TVII*$^{-/-}$), which lack most of E-selectin ligands (16). While *Fuc-TVII*$^{-/-}$ monocytes only partially promoted tumor cell transmigration, fixed *Fuc-TVII*$^{-/-}$ monocytes showed no effect (Figure 5G). These observations strongly indicate that the interaction between endothelial E-selectin and selectin ligands on monocytes support tumor cell transmigration, while soluble factors from monocytes further promote this process.

Tumor cell- and monocyte-induced endothelial cell retraction is E-selectin dependent

Activation of selectins by ligand-binding or antibody crosslinking is known to trigger “outside-in” signaling in endothelial cells and leukocytes, which induces migration of leukocytes through the endothelial cells (27,28). To elucidate the mechanism how monocyte-E-selectin interaction contributes to tumor cell transmigration we analyzed the cytoskeletal retraction of endothelial cells after 8 hours of co-culture with tumor cells with or without monocytes. Tumor cells or monocytes alone only slightly increased retraction of lung endothelial cells isolated from C57BL/6 mice as determined by Phalloidin-FITC staining (Figure 6A and C, D). The combination of tumor cells with monocytes strongly increased the number of retracting endothelial cells. However, we observed no cytoskeletal rearrangements in E-selectin deficient endothelial cells after co-culture with tumor cells or monocytes alone; nor with the combination of both cells (Figure 6B and D). These results support the conclusion that E-selectin-dependent endothelial activation mediated by selectin ligands on monocytes promotes endothelial transmigration of tumor cells.

E-selectin facilitates spontaneous lung metastasis of tumor cells without E-selectin ligands

We validated the role of E-selectin as facilitator of metastasis in a more clinically relevant model using a spontaneous lung metastasis assay using Lewis Lung carcinoma cells (LLC1) injected subcutaneously in a mouse flank. No major differences were observed in the primary tumor growth (data not shown). However, lung metastasis was significantly decreased in *E-sel*$^{-/-}$ mice compared to C57BL/6 mice (Figure 7A-B), further supporting the essential role of E-selectin in formation of pre-metastatic niche and thereby metastasis.

Discussion

The cross-talk between tumor cells and their host environment is essential in all steps of the metastatic cascade (4). Metastatic tumors often express aberrant glycan structures that are potential ligands for vascular selectins (1,3). E-selectin has been previously studied only as a direct receptor for tumor cell-endothelial adhesion (12,29). However, initial arrest of tumor cells is also likely caused by physical constrictions in small capillaries and therefore independent of active adhesion (30). This raised the question whether E-selectin affects the metastatic cascade by other means than via a direct tumor cell adhesion to the endothelium. Since we used tumor cells without endogenous E-selectin ligands, a direct tumor cell binding to E-selectin and thereby endothelial adhesion could be excluded. The observed reduction of both monocyte recruitment and engagement with tumor cells in the lungs of *E-sel*$^{-/-}$ mice support the role of E-selectin as a mediator of host cell interactions within the tumor microenvironment.

Markers of endothelial activation and inflammation are often up-regulated during the initial hours of intravascular tumor cell arrest in experimental metastasis models both in lungs and liver metastasis (12,29,31,32). Conversely reduction of endothelial activation diminished myeloid cell recruitment, tumor cell survival and metastasis (32-34). In line with these observations, we detected up-regulation of E-selectin, shortly after tumor cell arrest in the lung vasculature. While increased levels of endothelial activation markers VCAM-1 and ICAM-1 where detected in lungs of C57BL/6 mice no alterations of these adhesion molecules were detected in the lungs of *E-sel*$^{-/-}$ mice. Tumor cell-derived factors such as cytokines and chemokines together with physical factors like shear forces may trigger endothelial expression of E-selectin (31). We show that the tumor cell-derived chemokine CCL2 partially regulates vascular E-selectin induction by demonstrating that tumor cells with reduced CCL2 expression elicit weaker E-selectin expression. Chemokine production has been linked to E-selectin expression only in few studies in inflammatory models (35,36). In an atherosclerotic rat model the expression of CCL2 and adhesion molecules including E-selectin were up-regulated on the endothelium and associated with monocyte recruitment (35). The inhibition

of p38 MAPKs in TNF-α stimulated-endothelial cells resulted in reduced E-selectin and chemokine expression including CCL2, IL-8, and IL-6 (36). Rapid cytokine production (e.g. IL-1) was observed in an experimental liver metastasis model one hour after intra-splenic injection of Lewis lung carcinoma subline H-59 (29). We observed that tumor cell-induced endothelial activation additionally up-regulates CCL2 expression by endothelial cells and monocytes and thereby contributes to metastasis (Figure 7C).

CCL2 is a potent regulator of monocyte recruitment to metastasizing tumor cells and increased CCL2 levels at metastatic sites strongly correlate with metastasis (18-20). Furthermore, tumor cell-derived CCL2 activation of endothelial CCR2 induced vascular permeability and thereby assisting tumor cell extravasation (19). In this study, we observed decreased expression of CCL2 and CCR2 in lungs of *E-sel*$^{-/-}$ mice compared to C57BL/6 mice. We identified both monocytes and endothelial cells as the major source of CCL2 in the activated metastatic environment. These findings are in line with previous observations that stromal-derived CCL2 contributes to metastasis (18,20,21). Whether CCL2 induced endothelial activation is directly linked to E-selectin expression requires further investigation. Practically unaffected CCL2 expression in monocytes isolated from lungs of *E-sel*$^{-/-}$ mice might be a result of lacking activation. Previously it was reported that monocyte-HUVEC interactions resulted in increased expression of CCL2 and IL-8, suggesting that leukocyte adhesion to endothelial cells causes their activation and subsequent increase in chemokine production (37). Our results suggest that E-selectin is the initiator of endothelial activation which is substantial for myeloid cell-derived CCL2 secretion within the developing metastatic niche (Figure 7C).

Recruitment and activation of immune cells, especially inflammatory monocytes are strongly associated with enhanced metastatic colonization. Monocyte-secreted factors such as cytokines and chemokines assist intravascular tumor cells to survive and to extravasate from the circulation (11,15-19). We observed less recruitment of myeloid cells including macrophages, inflammatory monocytes and granulocytes to metastatic sites in lungs of *E-sel*$^{-}$

$^{/-}$ mice. The absence of E-selectin in combination with reduced CCL2 levels is likely impeding efficient capturing and firm adhesion of recruited monocytes.

Previous studies revealed that selectin-mediated leukocyte interactions strongly support metastasis during early stages (15,16,22). This work provided evidence that enhanced monocyte-assisted tumor cell transmigration is dependent on endothelial E-selectin expression and on selectin ligands presented by monocytes. The finding that fixed monocytes displaying only selectin ligands on their surfaces were able to induce trans-endothelial migration of tumor cells, strongly indicates that E-selectin ligation is necessary for this process.

Leukocyte binding to E-selectin is known to induces signaling pathways which trigger the extravasation cascade in endothelial cells via “outside-in” signaling engaging MAPK, PLC-γ, Erk/Src or MLC/p38 pathways (27,38). These signaling events promoted increased monolayer permeability through a disruption of VE-cadherin/β-catenin complex or formation of stress fibers which both facilitated trans-endothelial migration (10,39). Here we observed that monocytes assist tumor cells to induce cytoskeletal retraction in endothelial cells in an E-selectin dependent manner. In addition, depletion of monocytes before tumor cell injection prevented increase in lung permeability strongly suggesting that E-selectin and monocytes are important regulators of pulmonary vascular permeability in response to tumor cells. Recently, it was reported that factors released from a primary tumor up-regulate expression of E-selectin and focal adhesion kinase to form hyperpermeable foci where tumor cells can extravasate (14). We also detected reduced pulmonary metastasis in tumor bearing *E-sel*$^{-/-}$ mice which further confirms the role of E-selectin during metastasis. Taken together, E-selectin-mediated interactions between endothelium and leukocytes in combination with induced CCL2 expression critically affect the shaping of a metastatic niche which facilitates tumor cell extravasation and initiates successful development of secondary tumors.

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Authorship contribution

I.H. planned, conducted and analyzed experiments and wrote the manuscript. M.R. conducted experiments. V.H., S.G., A.B. and S.S. provided technical assistance with flow chamber experiments. L.B designed the research, planned and performed in vivo experiments, analyzed data and wrote the manuscript.

Conflict-of-interest disclosure

Authors declare no conflict of interest.

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Figure legends

Figure 1. Tumor cells with no E-selectin ligands metastasize in E-selectin dependent manner. Mice were intravenously injected with tumor cells and lungs analyzed for metastasis. **A)** Representative images of dissected lungs 28 days after MC-38GFP cell injection. **B)** Quantification of metastatic foci. Representative images of lungs of mice injected with 3LL **(C)** or B16-BL6 cells **(E)** after 14 days. Quantification of metastatic foci in 3LL **(D)** and B16-BL6 injected mice **(F)**. *; $p<0.05$; ***; $p<0.001$.

Figure 2. E-selectin dependent leukocyte recruitment to the metastatic organ. A-C) Flow cytometry analysis of lung homogenates from C57BL/6 and *E-sel*$^{-/-}$ mice at 24 or 48 hours p.i. were compared to lungs of naïve mice, respectively. The number of total infiltrated leukocytes (CD45^{+}), inflammatory monocytes (Ly6C^{high}) and macrophages (F4/80^{+}) was normalized to 1'000 endothelial cells (CD31^{+}). **D-F**, Lung analysis for macrophages (F4/80^{+}) **(D)** and granulocytes (Ly6G^{+}) **(E)** at 16 and 24 hours p.i. were compared to naïve lungs. **F)** Representative images of F4/80^{+} cells (red) in lungs of C57BL/6 and *E-sel*$^{-/-}$ mice at 16 or 24 hours p.i. compared to naïve lungs of respective strain. Nuclei (blue) are stained with DAPI. Scale bar = 20 µm. **G)** Quantification of tumor cells in lung sections prepared from mice 16 or 24 hours p.i. Data are presented as mean ± SEM. *; $p<0.05$.

Figure 3. Tumor cell-induced E-selectin expression facilitates specific leukocyte association with tumor cells at metastatic sites. A) E-selectin expression in the whole lungs isolated from C57BL/6 mice at 6, 12 and 24 hours p.i. were compared to lungs of naïve mice. Expression levels determined by real-time PCR were normalized to GAPDH expression. Data are expressed as mean ± SEM. ***; $p<0.001$ by ANOVA. **B)** Confocal microscopy images of E-selectin expression (red) in the vicinity of tumor cells (green) on lung sections at indicated times p.i. Nuclei (blue) were stained with DAPI. Bar = 10 µm. **C)** Confocal microscopy of lung sections 6 h p.i. Tumor cells (green) were associated with CD11b^{+} cells (white) in the vicinity of E-selectin^{+} (red) endothelial cells. Nuclei (blue) were stained with DAPI. **D)** Analysis of tumor cell-leukocyte association in lungs of C57BL/6 and

E-sel$^{-/-}$ mice 16 and 24 hours p.i. Inset: Image of a tumor cell (green) in contact with F4/80^{+} cells (red) in lungs of mice 16 hours p.i. Nuclei (blue) are stained with DAPI. Bar = 10 µm. **E)** Microscopic image of MC-38GFP cells (green) attached to an endothelial monolayer in a microfluidic device (left panel). Representative images of tumor cells (green) adherent on endothelial cells derived from C57BL/6 (middle panel) and *E-sel*$^{-/-}$ mice (right panel) and recruited monocytes (red) 50 minutes after initiation of monocyte perfusion. **F)** Number of MC-38GFP cells associated with monocytes on endothelial cells at 15, 50 and 90 minutes after perfusion with monocytes was induced in a microfluidic device. **G)** Number of monocytes associating with a single tumor cell attached to endothelial cell monolayers at indicated times. **H)** Number of tumor cells quantified on lung sections from mice 16 or 24 hours p.i. Data are presented as mean ± SEM. *; p<0.05; ***; p<0.001.

Figure 4. E-selectin expression is induced through tumor cell-derived CCL2 and further endothelial activation facilitates increase in CCL2 chemokine pool in metastatic lungs. A-D) Total RNA was isolated from the whole lungs of untreated C57BL/6 and *E-sel*$^{-/-}$ mice and 6, 12 and 24 hours p.i. Expression levels of VCAM-1 **(A)**, ICAM-1 **(B)**, CCL2 **(C)** and CCR2 **(D)** were analyzed by real-time PCR and normalized to GAPDH. **E-G)** Total RNA was isolated from the whole lungs of untreated C57BL/6 mice and 6 hours p.i. with MC-38GFP and MC-38GFP CCL2 KD cells, respectively. Expression levels of E-selectin **(E)**, VCAM-1 **(F)**, CCL2 **(G)** were analyzed by real-time PCR and normalized to GAPDH. *; p<0.05; **; p<0.01. **H-I)** CCL2 protein levels in the perfused lung homogenate of untreated C57BL/6, *E-sel*$^{-/-}$ and *Ccl2*$^{-/-}$ mice 6 hours **(H)** and 12 hours p.i. **(I)** detected by cytokine bead array. CCL2 protein levels are normalized to total protein content. n = 3; *; p<0.05; **; p<0.01; ***; p<0.001 by ANOVA. **J)**, CCL2 expression levels in pulmonary endothelial cells and inflammatory monocytes sorted from digested lungs of untreated C57BL/6 and *E-sel*$^{-/-}$ mice and 12 hours p.i. Data were analyzed by real-time PCR and normalized to GAPDH. Data are presented as mean ± SEM. n = 3; **; p<0.01 by Student's t-test.

Figure 5. E-selectin expression promotes increased lung vascular permeability and facilitates tumor cell transmigration in monocyte-derived selectin ligand dependent manner A) Macroscopic images of lungs from untreated C57BL/6 and *E-sel*$^{-/-}$ mice and 24 hours p.i. injected with Evans blue. **C)** Macroscopic images of lungs from untreated or clodronate liposome treated C57BL/6 mice 24 hours p.i. injected with Evans blue. **B** and **D**) Spectrophotometric quantification of Evans blue extracted from perfused lungs. **E)** Number of MC-38GFP cells transmigrated through lung endothelial cells derived from C57BL/6 and *E-sel*$^{-/-}$ mice in the absence or presence of monocytes after 16 hours of co-culture. **F)** Representative images of transmigrated MC-38GFP cells (green) counted on the lower side of the transwell membrane. **G)** Number of MC-38GFP cells transmigrated through lung endothelial cells derived from C57BL/6 mice in the presence of monocytes. Normal (unfixed) monocytes were compared to fixed monocytes derived from C57BL/6 or *Fuc-TVII*$^{-/-}$ mice after 16 hours of co-culture. Data are presented as mean ± SEM. **; $p<0.01$; ***; $p<0.001$; ns = not significant.

Figure 6. E-selectin-dependent endothelial cell retraction is induced by tumor cells and monocytes. A-B) Representative images of Phalloidin-FITC stained cytoskeleton (green) of endothelial cells, derived from C57BL/6 **(A)** or *E-sel*$^{-/-}$ **(B)** mice, after co-culture with MC-38 cells (TC, red) and/or monocytes for 8 hours. Arrowheads indicate retracting endothelial cells. Bar = 50 µm. **C)** High power images of endothelial cells from C57BL/6 mice with MC-38 cells (red) and monocytes, showing endothelial cell retraction stained by Phalloidin-FITC (arrows) and the adjacent monocytes (arrowheads) after co-culture for 8 hours. Nuclei (blue) are stained with DAPI. Bar = 20 µm. **D)** Quantification of endothelial cell retraction stained with Phalloidin-FITC of untreated endothelial cells from C57BL/6 or *E-sel*$^{-/-}$ mice (ctrl); or after co-culture with MC-38 cells (TCs) and/or monocytes (monos). Data are presented as mean ± SEM. ***; $p<0.001$ by ANOVA with the post-hoc Bonferroni multiple comparison test.

Figure 7. E-selectin facilitates spontaneous lung metastasis of Lewis lung carcinoma. **A)** Representative images of dissected lungs from C57BL/6 and *E-sel*$^{-/-}$ mice and H&E stained lung sections. **B)** Number of metastatic foci in lungs of tumor bearing C57BL/6 and *E-sel*$^{-/-}$ mice 30 days after subcutaneous injection of LLC1 cells. **; $p<0.01$. **C) Scheme of E-selectin-mediated interactions contributing to metastasis. 1)** Tumor cells in circulation interact with platelets and leukocytes, eventually forming emboli that get trapped in the microvasculature. Shear stresses together with tumor-cell derived factors, e.g. CCL2, activate the endothelium resulting in E-selectin expression. **2)** E-selectin on the endothelial cell surface mediates adhesion of leukocytes, which are recruited by tumor- and host-derived chemokines such as CCL2. Upon ligation of E-selectin by leukocytes “outside-in” signaling is induced both in leukocytes and in endothelial cells starting the extravasation cascade. **3)** This includes firm adhesion of leukocytes to endothelium via integrin and VCAM-1/ICAM-1 interaction as well as cytoskeletal remodeling and retraction in endothelial cells. Ultimately tumor cells extravasate through the permeable vasculature.

Figure 1

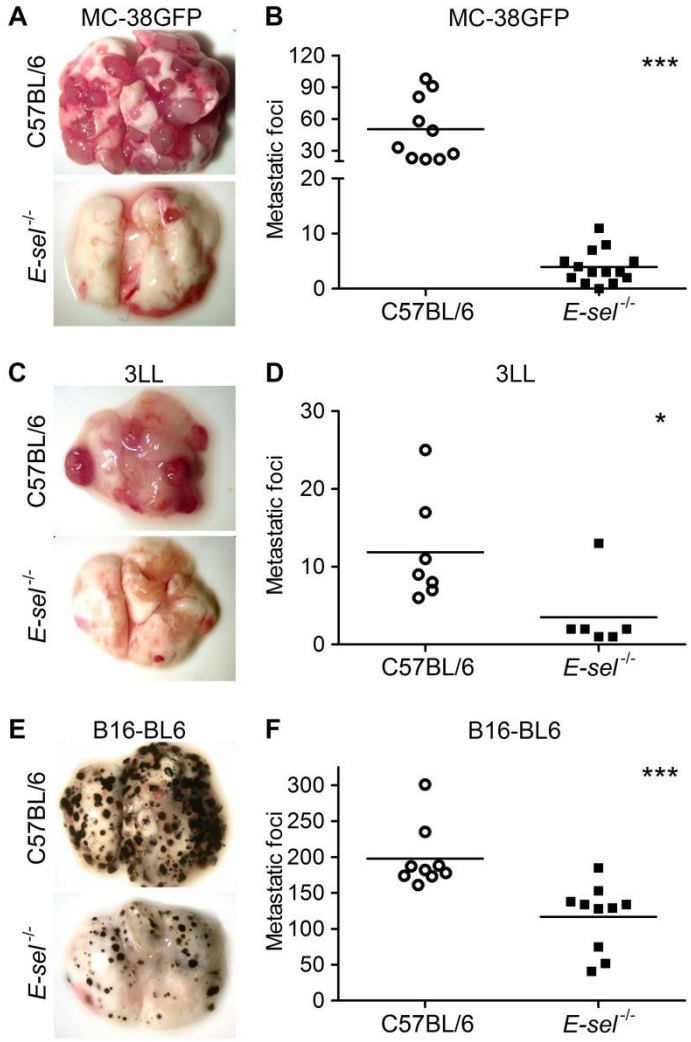
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Figure 2

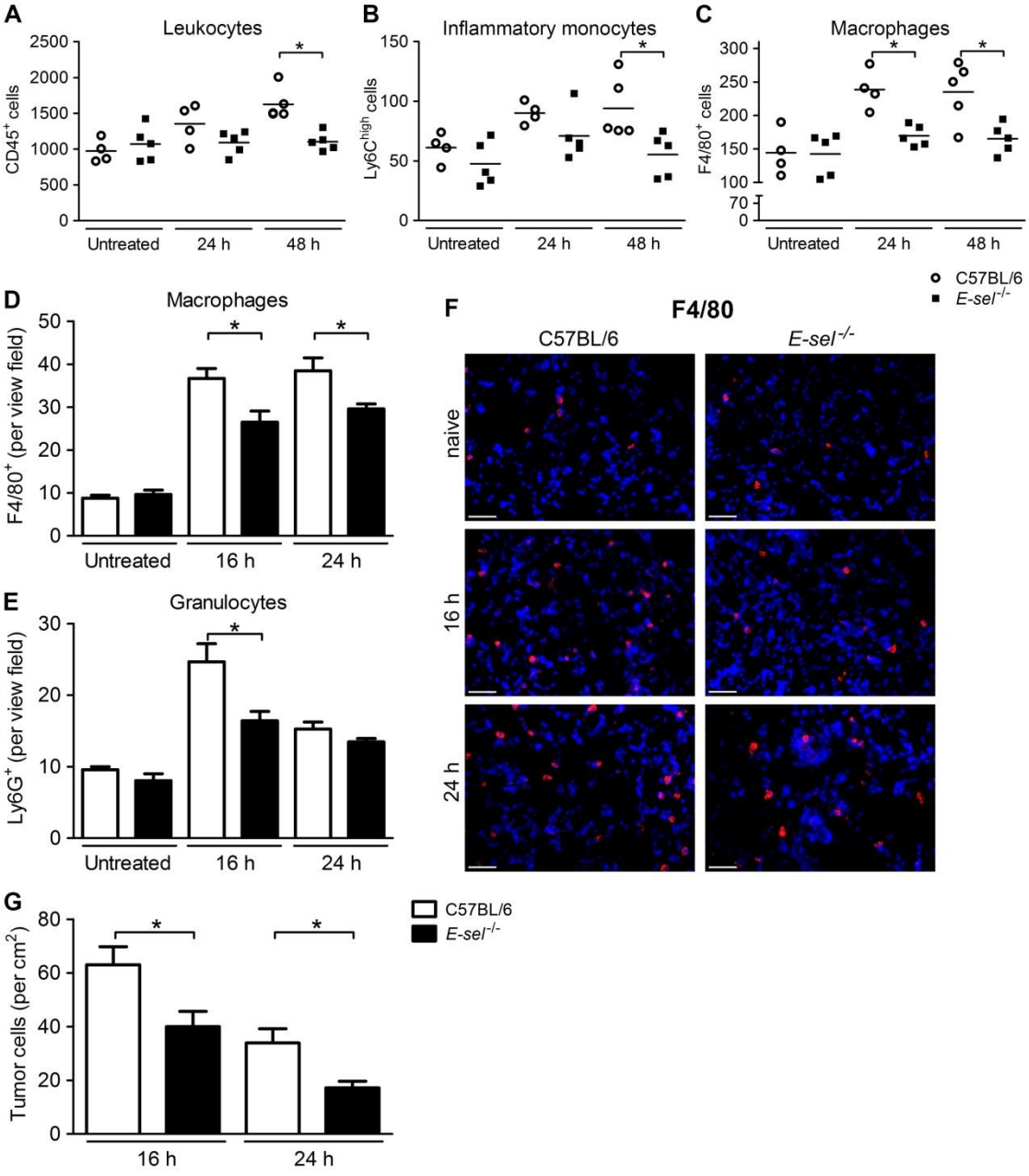
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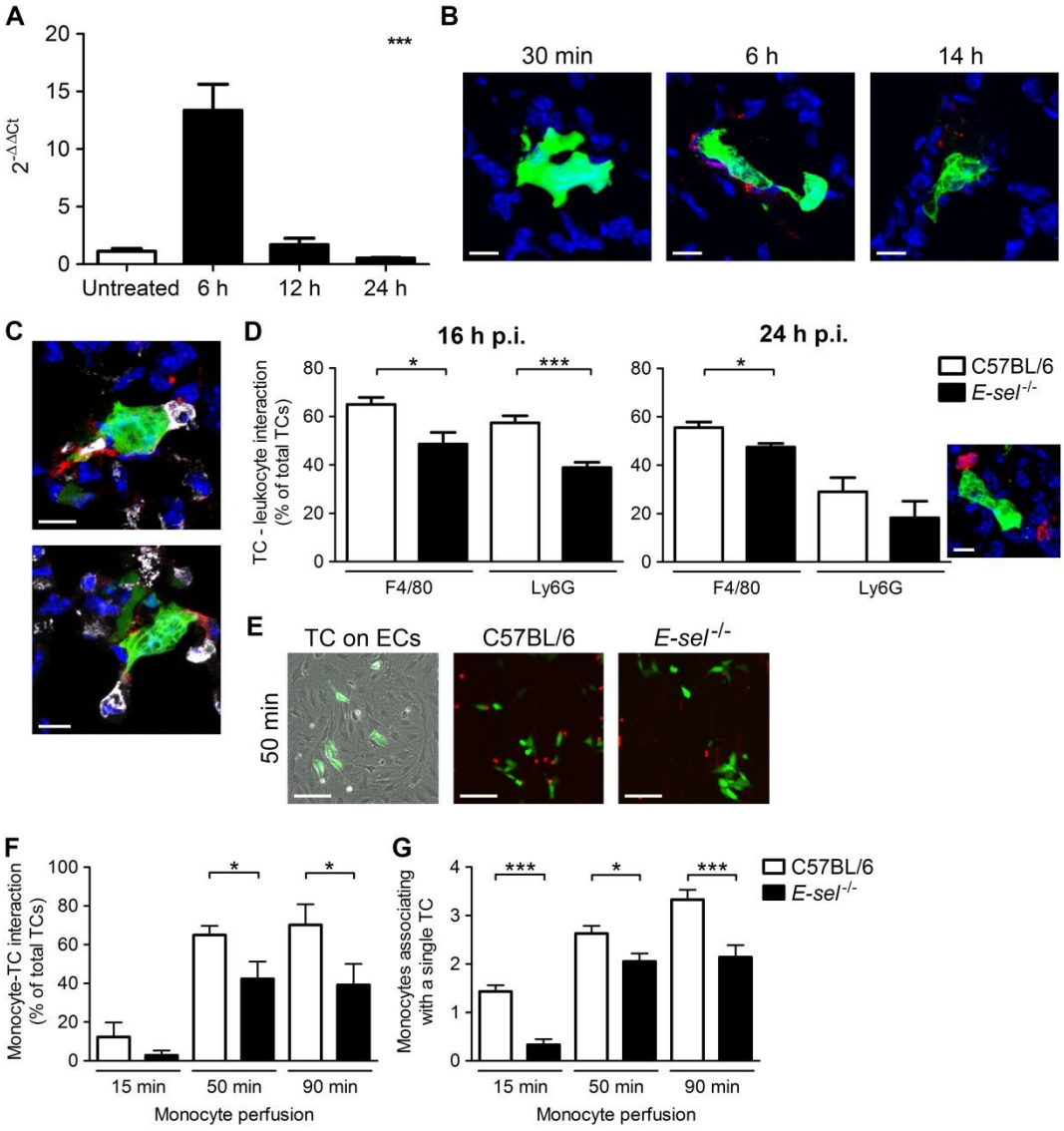
Figure 3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TC - leukocyte interaction (% of total TCs)
F4/80
Ly6G
TC on ECs
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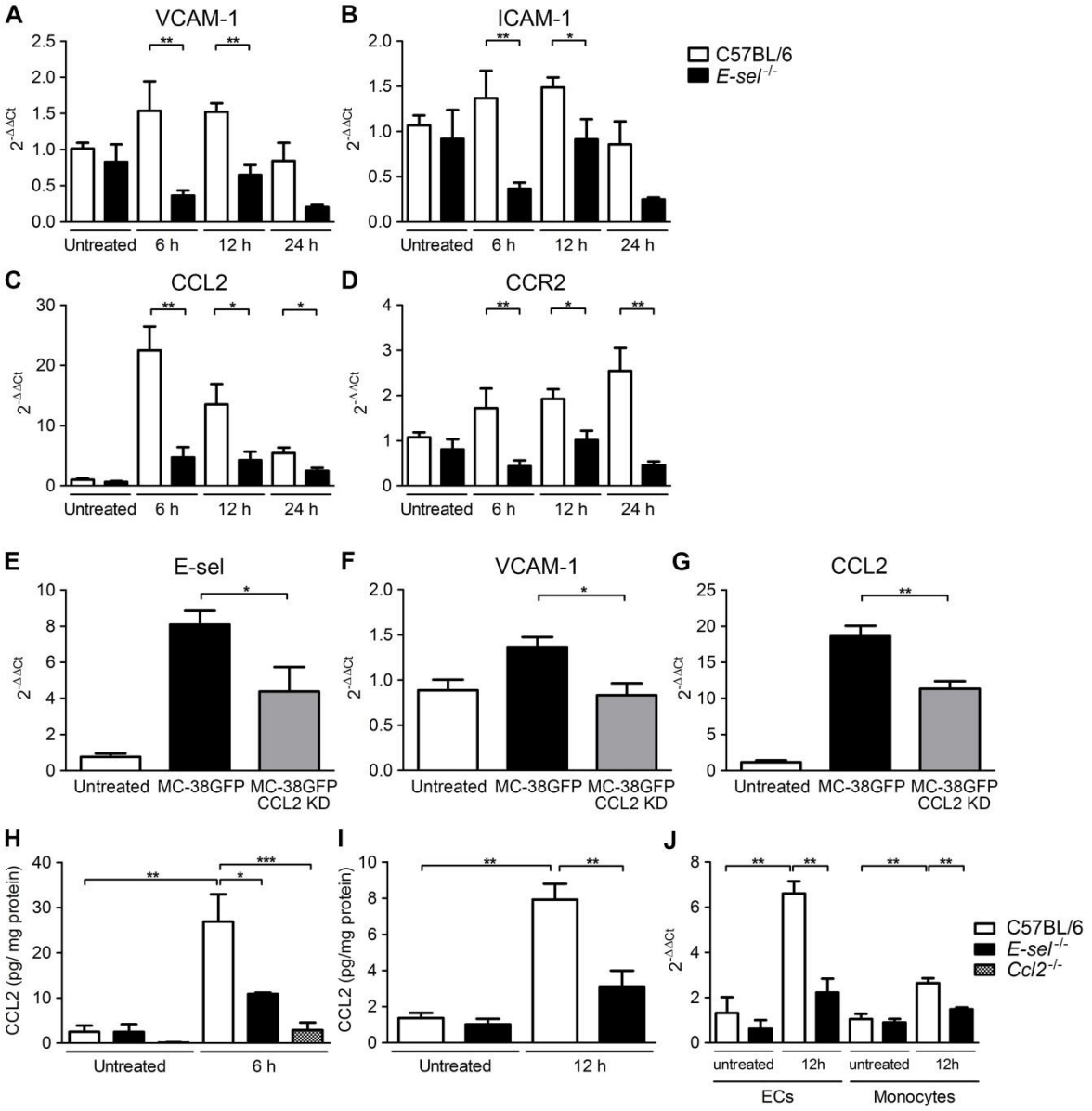
Monocyte-TC interaction (% of total TCs)
Monocytes associating with a single TC
15 min
50 min
90 min
Monocyte perfusion

Figure 4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24 h
E
E-sel
F
VCAM-1
G
CCL2
Untreated
MC-38GFP
MC-38GFP CCL2 KD
H
CCL2 (pg/ mg protein)
I
CCL2 (pg/mg protein)
J
Ccl2-/-
untreated
12h
ECs
Monocytes

Figure 5

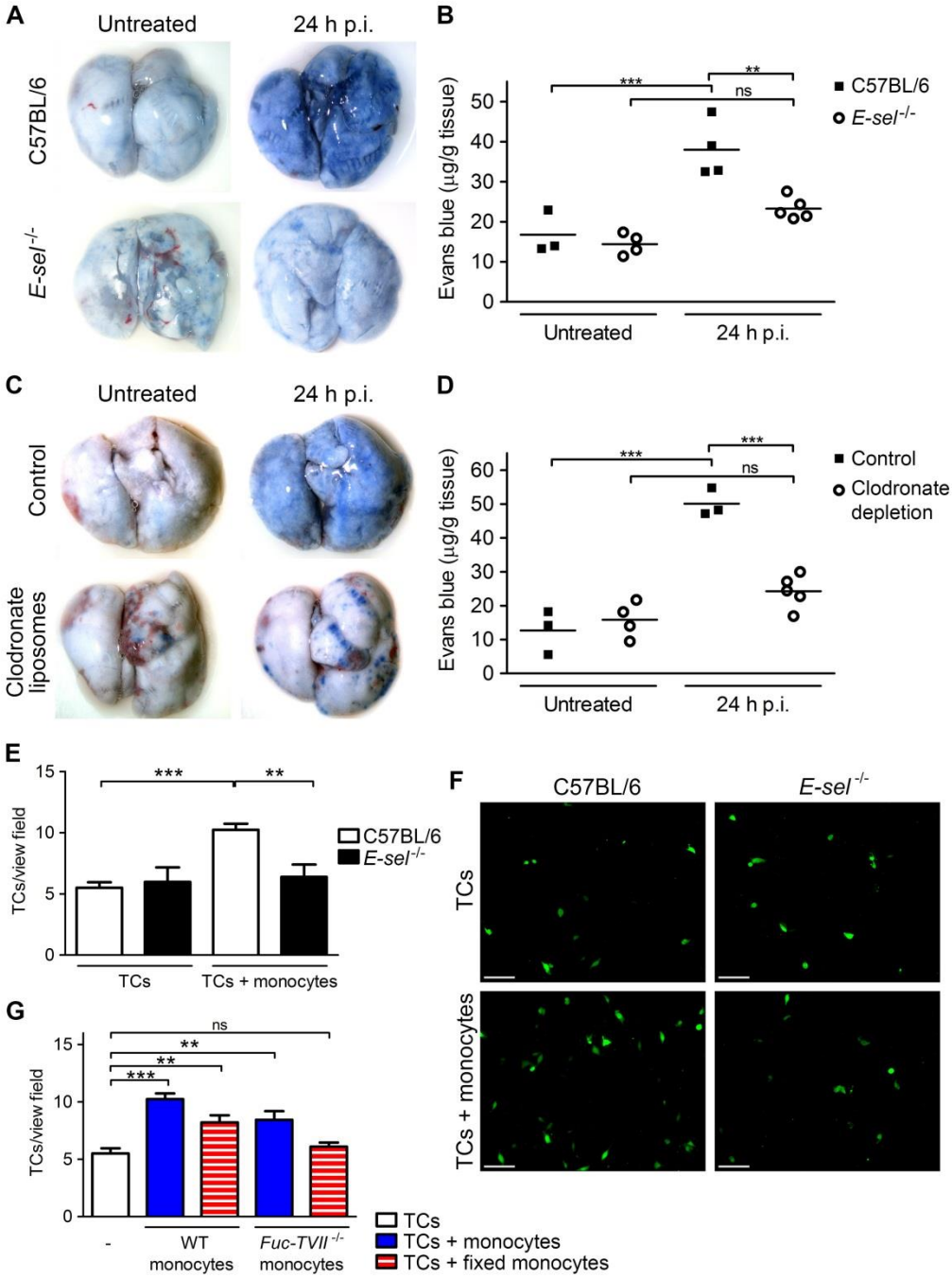

A
Untreated
24 h p.i.
C57BL/6
E-sel-/-
B
Evans blue (µg/g tissue)
***
**
ns
C57BL/6
E-sel-/-
Untreated
24 h p.i.
C
Untreated
24 h p.i.
Control
Clodronate liposomes
D
Evans blue (µg/g tissue)
***
***
ns
Control
Clodronate depletion
Untreated
24 h p.i.
E
TCs/view field
***
**
C57BL/6
E-sel-/-
TCs
TCs + monocytes
F
C57BL/6
E-sel-/-
TCs
TCs + monocytes
G
TCs/view field
ns
**
**
***
-
WT monocytes
Fuc-TVII-/- monocytes
TCs
TCs + monocytes
TCs + fixed monocytes

Figure 6

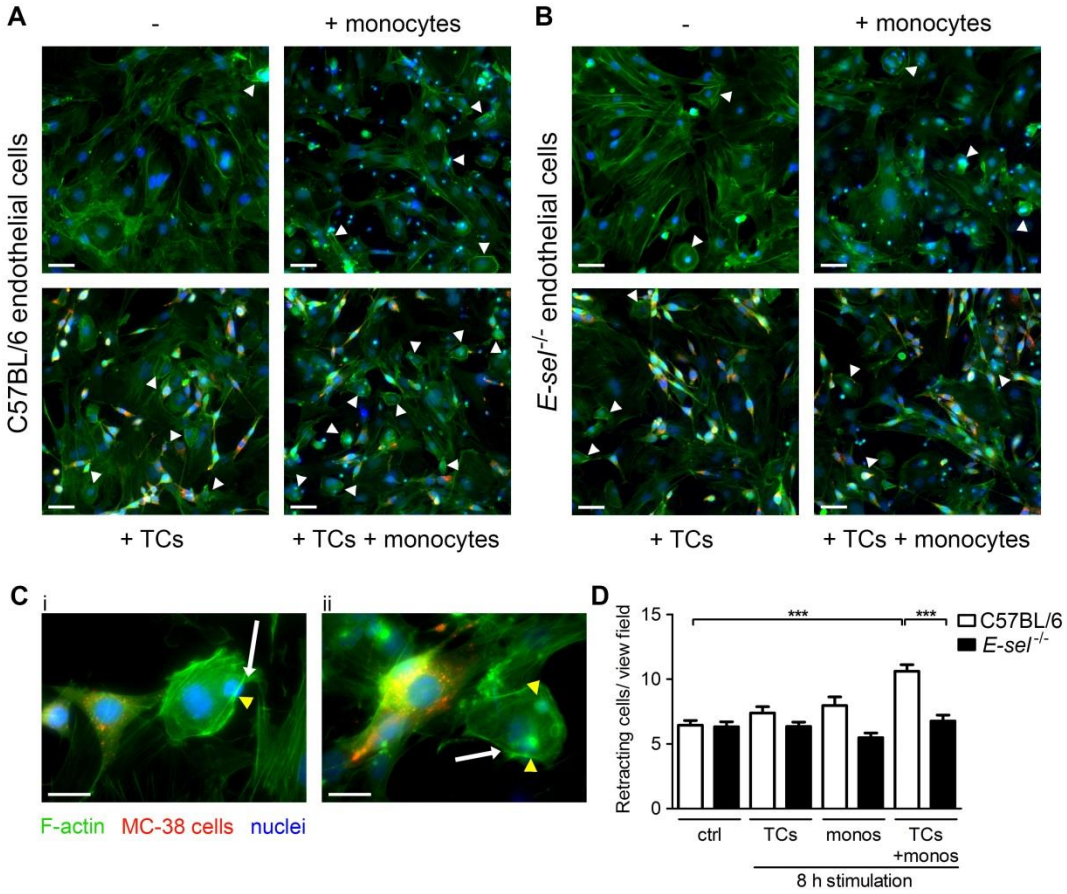
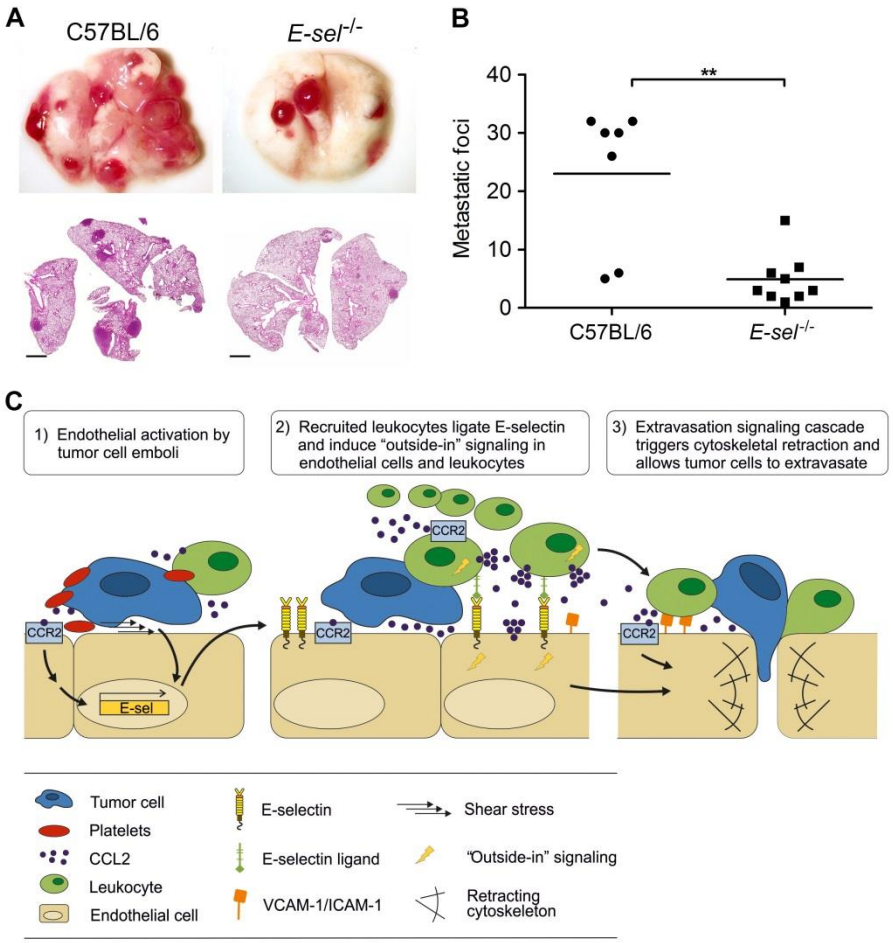

A
-
+ monocytes
C57BL/6 endothelial cells
+ TCs
+ TCs + monocytes
B
-
+ monocytes
E-sel-/- endothelial cells
+ TCs
+ TCs + monocytes
C
i
ii
F-actin MC-38 cells nuclei
D
Retracting cells/ view field
15
10
5
0
***
***
C57BL/6
E-sel-/-
ctrl
TCs
monos
TCs +monos
8 h stimulation

Figure 7


A
C57BL/6
E-sel-/-
B
Metastatic foci
40
30
20
10
0
**
C57BL/6
E-sel-/-
C
1) Endothelial activation by tumor cell emboli
2) Recruited leukocytes ligate E-selectin and induce "outside-in" signaling in endothelial cells and leukocytes
3) Extravasation signaling cascade triggers cytoskeletal retraction and allows tumor cells to extravasate
CCR2
E-sel
Tumor cell
Platelets
CCL2
Leukocyte
Endothelial cell
E-selectin
E-selectin ligand
VCAM-1/ICAM-1
Shear stress
"Outside-in" signaling
Retracting cytoskeleton

Supplementary Material

E-selectin-mediated monocyte adhesion and activation of the pulmonary endothelium induce vascular permeability and promote metastasis

Irina Häuselmann, Marko Roblek, Volker Huck, Sandra Grässle, Alexander Bauer, Stefan W. Schneider, Lubor Borsig

Inventory of Supplemental Information

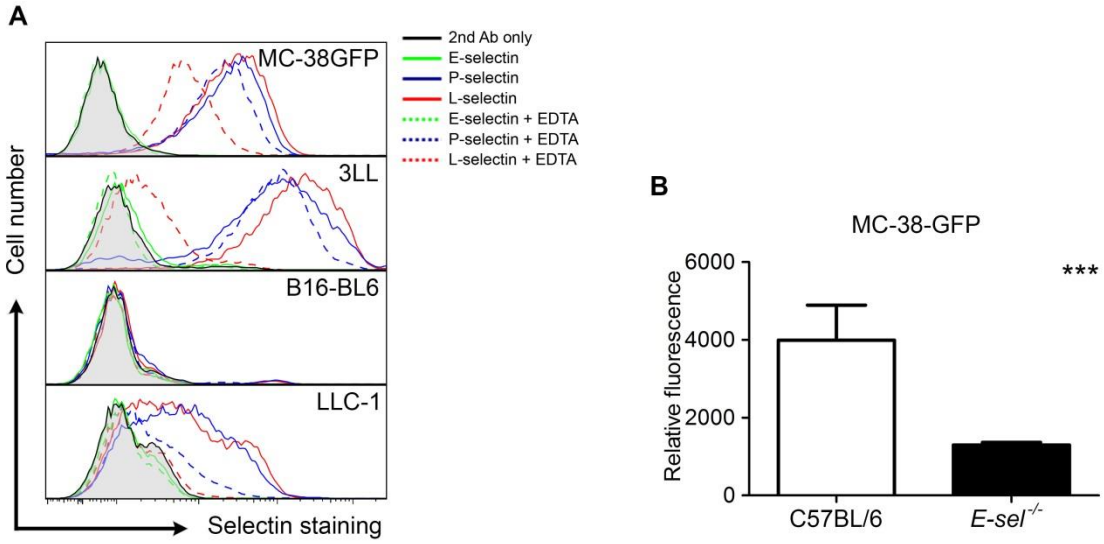


Figure S1. A) Murine tumor cells do not express E-selectin ligands. Representative histograms of E-, P-, and L-selectin binding to MC-38GFP, 3LL, B16-BL6 and LLC-1 cells using recombinant mouse selectins analyzed by flow cytometry. Filled areas represent control profiles with secondary antibodies only. Solid lines represent selectin-stained cells. Dashed lines represent selectin staining in the presence of 10 mM EDTA (negative control). **B) Metastatic tumor burden in mice injected with MC-38GFP cells.** Quantification of metastatic load by measurement of GFP fluorescence in lung homogenates of C57BL/6 and E-sel$^{-/-}$ mice 28 days after injection of MC-38GFP cells as previously described (1).

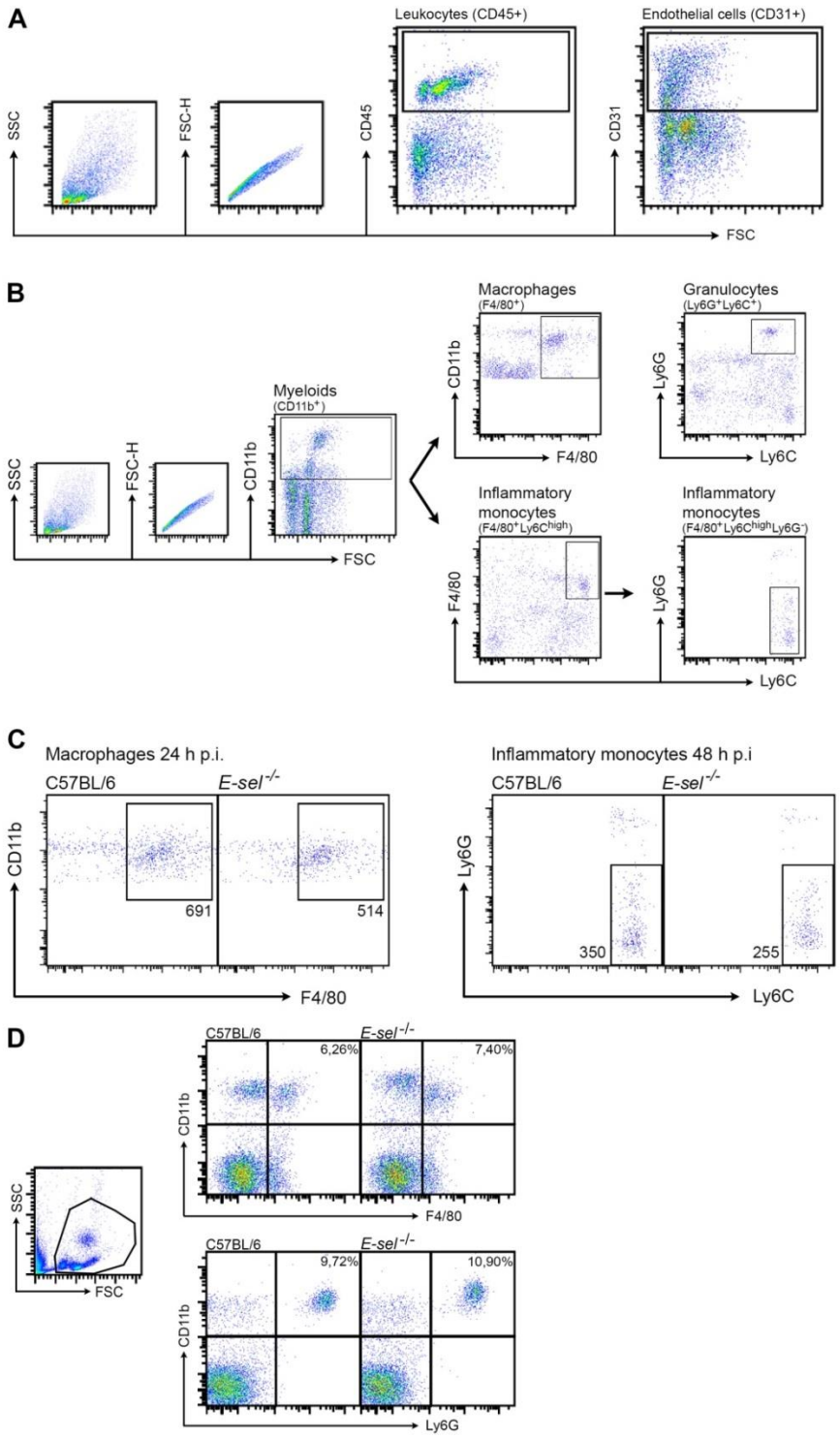


Figure S2 A) Gatig strategy for leukocyte populations in lungs after tumor cell injection. Leukocyte ($CD45^+$) infiltration was quantified in relation to pulmonary endothelial cells ($CD31^+$) used as an internal reference. **B)** Gating strategy for the quantification of leukocyte subpopulations infiltrated into lungs of C57BL/6 and E-$sel^{-/-}$ mice after MC-38GFP injection. **C)** Representative flow cytometry plots of macrophages (left panel) and inflammatory monocytes (right panel) in lungs of mice at indicated time points after MC-38 GFP cell injection**. D) Peripheral mononuclear blood cells of C57BL/6 and E-$sel^{-/-}$ mice showed no difference.** Representative flow plots of peripheral mononuclear blood cells in C57BL/6 and E-$sel^{-/-}$ mice.

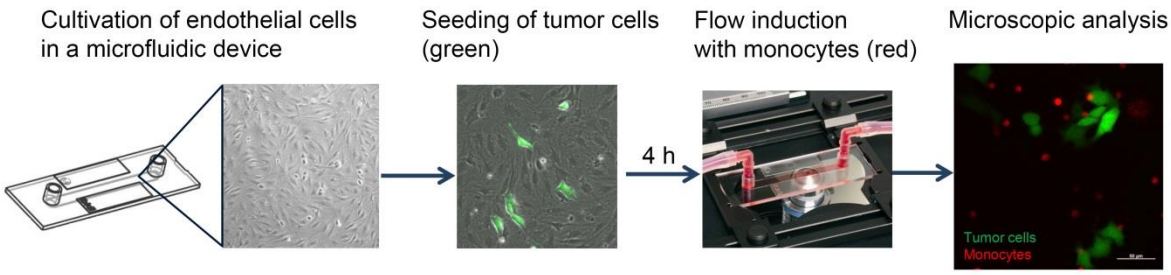


Figure S3. Microfluidic system work flow. Primary lung endothelial cells (ECs) were cultivated on a microfluidic slide, MC-38GFP cells were seeded on the confluent EC layer and 4 hours later the perfusion with monocytes in HEPES buffered Ringer solution supplemented with 25% washed red blood cells was started (2 dyne/cm^2).

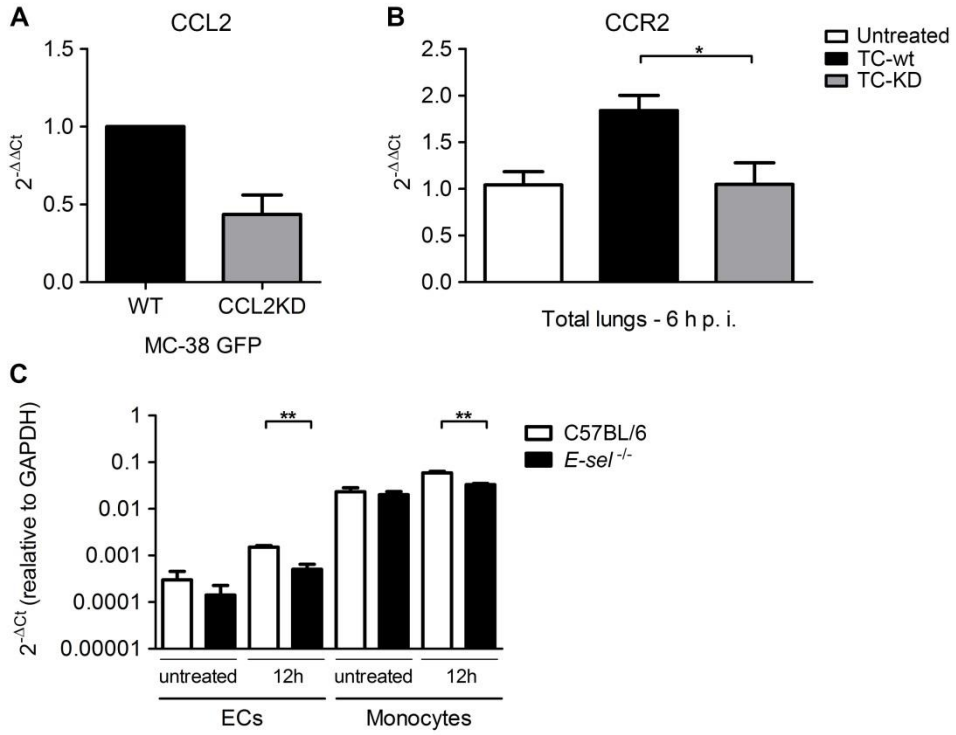


Figure S4. Tumor-cell derived CCL2 contributes to enhanced CCR2 expression in metastatic lungs. A) CCL2 expression levels in cultured MC-38GFP cells (WT) and MC-38GFP with silenced CCL2 (CCL2KD). Expression levels were analyzed by real-time PCR and normalized to GAPDH. **B)** CCR2 expression in whole lungs isolated from untreated C57BL/6 and 6 hours after WT-MC-38GFP or CCL2KD-MC-38GFP cell injection. **C)** CCL2 expression levels relative to GAPDH expression in sorted endothelial cells (ECs), and monocytes from untreated and tumor cell-injected mice 12 hours p.i. Data are presented as mean ± SEM. n= 3; *; $p<0.05$; **; $p<0.01$ by Student's t-test.

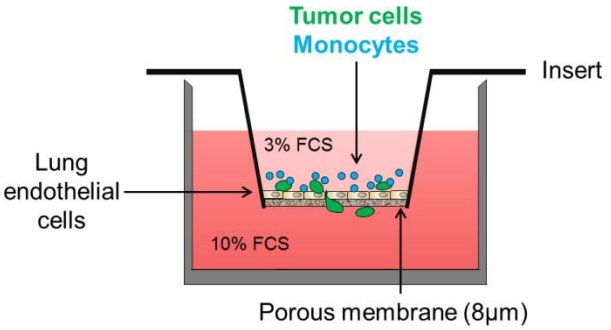


Figure S5. Transmigration assay scheme. Tumor cells and bone marrow purified monocytes ($CD115^+$ cells) in 3% FCS media are seeded to a confluent endothelial monolayer on a porous membrane of a transwell insert. Transmigrated tumor cells on the lower side of the membrane in the lower well containing 10% FCS media were counted.

Table S1.

Primers	Sequences 5`-3`	Fragment size (bp)	Tm (°C)
E-sel	CGCCAGAACAACAATTCCAC ACTGGAGGCATTGTAGTACC	157	60
CCL2	TTAACGCCCCACTCACCTGC TGGGGTCAGCACAGACCTCTC	153	60
CCR2	GCAAGTTCAGCTGCCTGCAAA GTATGCCGTGGATGAACTGAGGT	141	60
ICAM-1	CCCCGCAGGTCCAATTCACA CCAAGCAGTCCGTCTCGTCC	162	60
VCAM-1	GCGGTCTTGGGAGCCTCAAC GTGACTCGCAGCCCGTAGTG	145	60
GAPDH	CCCAGCAAGGACACTGAGCAA GTGGGTGCAGCGAACTTTATTGATG	161	60

Supplemental Experimental Procedures

Selectin binding to tumor cells

Selectin ligand staining on tumor cells was performed as described previously (2). Briefly, mouse selectin-Fc chimeras (L-, P-, and E-selectins) were pre-complexed with biotinylated goat anti-human antibody (1:100; Sigma-Aldrich) prior to incubation with tumor cells. Selectin binding was detected with Streptavidin-CyChrome (BD Biosciences). Control samples were incubated with selectin chimeras in the presence of 10 mM EDTA. Data were acquired on a BD FACSCanto II flow cytometer (BD Biosciences) and analyzed with the Flow Jo v8.8.4 software (Tree Star).

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DISCUSSION

The interplay between tumor cells and their host environment is essential for all steps of the metastatic cascade, starting with the separation of tumor cells from the primary tumor mass and continuing with steps such as intravasation, survival in the circulation, adhesion to the microvasculature and ultimately tumor cell exit into the parenchyma (1). In this study we describe a novel mechanism whereby E-selectin exerts pro-metastatic effects via chemokine-assisted interactions with leukocytes at the metastatic site.

1. E-selectin enables tumor cell arrest and adhesion in the microvasculature

During the last decades several mechanisms concerning how selectins facilitate the metastatic process have been explored and demonstrate that selectins are important promoters of metastasis. E-selectin in particular has been strongly associated with metastasis and poor disease outcome in cancer patients which has been confirmed with experimental studies (2,3). E-selectins role as a promoter of metastasis has mostly been attributed to its binding to E-selectin ligands presented on tumor cells thereby enabling tumor cell arrest in the microvasculature, a prerequisite for efficient extravasation (4). Numerous experiments *in vitro* demonstrate that tumor cells bind to endothelial E-selectin under flow conditions (5-7) and the overexpression of selectin ligands on tumor cells resulted in increased metastasis *in vivo* (8,9). However, experimental data indicate that the initial arrest of tumor cells probably does not depend on active adhesion and may actually occur due to physical trapping of tumor cells in small capillaries (10). Lung metastasis assays have demonstrated that tumor cell arrest in the lung microvasculature seems to be an event occurring prior to endothelial activation and E-selectin up-regulation (11). This observation has raised the question to what extent E-selectin is actually involved during tumor cell arrest and whether E-selectin affects the metastatic cascade by other means. Our study sought to decipher the contribution of E-selectin to metastasis apart from its adhesive interactions with tumor cells by analyzing the effect of E-selectin on the early metastatic environment.

2. Early E-selectin-mediated processes promote metastasis

We showed that experimental lung metastasis of tumor cells without E-selectin ligands was attenuated in E-selectin deficient mice. Using tumor cells without endogenous E-selectin ligands enabled us to exclude direct tumor cell-E-selectin adhesion as a promoter of metastasis in our experimental set up. Therefore, the observed reduction of metastatic nodules in absence of E-selectin indicates other activities of E-selectin during metastasis. An up-regulation of E-selectin has been reported in experimental lung metastasis models as an early event, occurring only a few hours after tumor cells are injected into the tail vein and diminishing within one day (11). In line with this data, we found that E-selectin mRNA and protein levels are up-regulated shortly after tumor cell injection which suggests that E-selectin plays a role soon after tumor cell emboli arrest in the lungs. The number of observed tumor cells in the lung was already lower within less than a day in the absence of E-selectin. This led us conclude that E-selectin is involved during communication within the metastatic microenvironment and thereby supports tumor cells during early metastatic processes such as tumor cell extravasation. The up-regulation of E-selectin in the lungs was accompanied by increased VCAM-1 and ICAM-1 levels which are additional markers of endothelial activation elicited by tumor cell emboli. E-selectin deficient lungs showed decreased VCAM-1 and ICAM-1 levels upon tumor cell injection demonstrating minimal responsiveness of the endothelium to activation triggers.

Markers of endothelial activation and inflammation are up-regulated only several hours after intravascular tumor cell arrest in experimental metastasis models (11-14). The inhibition of endothelial VCAM-1 and vascular adhesion protein-1 (VAP-1) has been recently reported to result in decreased myeloid cell recruitment and diminished tumor cell survival (14). Blocking endothelial activation had metastasis reducing effects (15,16). A possible explanation for the less activated endothelium observed in the absence of E-selectin might be a loss of signaling through pathways which are normally induced in stromal cells and contribute to an inflammatory environment.

Endothelial expression of E-selectin in response to tumor cells can be triggered by tumor cell emboli-produced shear forces or tumor cell-derived factors such as cytokines or chemokines (17). In this thesis we provide evidence that the tumor cell-derived chemokine CCL2 contributes to E-selectin induction on the pulmonary vasculature by demonstrating that tumor cells with reduced CCL2 expression elicit weaker E-selectin up-regulation in lungs than wild type tumor cells. The exact mechanism behind CCL2-mediated E-selectin up-regulation is currently unknown. Chemokine production and E-selectin expression have been linked in several studies. One such study demonstrated that in a rat model of hyperhomocysteinemia, CCL2 and adhesion molecules including E-selectin were up-regulated and mediated the adhesion of monocytes to the aortic endothelium (18). p38 MAPKs have been reported to induced E-selectin expression (19) and inhibition of p38 MAPKs in stimulated endothelial cells led to reduced E-selectin and chemokine and cytokine expression including IL-8, CCL2 and IL-6 (20). However, no detailed mechanistic pathway concerning the up-regulation of E-selectin via CCL2 has been established.

3. A link between E-selectin and the chemokine CCL2 during metastasis

Numerous studies have reported that CCL2 regulates monocyte recruitment to metastasizing tumor cells (21-23) and that elevated CCL2 levels at metastatic sites are strongly associated with metastasis of several cancer types *in vivo* (21,24,25). Both tumor cell- and stromal cell-derived CCL2 have been reported to recruit inflammatory monocytes to the lungs at early stages in various metastasis models (21,24). One possible explanation concerning CCL2s pro-metastatic activity is that it may interact with endothelial CCR2 thereby inducing increased vascular permeability that favors tumor cell extravasation (21).

In our study CCL2 and CCR2 levels were both increased in C57BL/6 lungs early after tumor cell injection but only minimal changes were detected when E-selectin was absent. Tumor cell injection into mice without endogenous CCL2 expression only revealed a minor increase of CCL2 protein levels in lungs compared to C57BL/6 and E-selectin deficient mice. Given the comparable number of tumor cells in lungs of C57BL/6, E-selectin and CCL2 deficient

mice at this early time point the small CCL2 increase in CCL2 deficient mice corresponds to the tumor cell-derived CCL2. This suggests that besides tumor cells endogenous host cells contribute to CCL2 production in C57BL/6 and E-selectin deficient mice. Here, we identified stromal cells as the predominant source of CCL2 in response to tumor cell injection by showing that inflammatory monocytes as well as endothelial cells strongly provided the metastatic environment with CCL2. Its expression was also diminished in these cell types in the absence of E-selectin causing the reduced CCL2 pool in E-selectin deficient lungs after tumor cell injection. Host-derived CCL2 may be secreted as a result of endothelial activation and E-selectin is required for proper endothelial CCL2 up-regulation in response to tumor cell emboli. Whether E-selectin is directly linked to signaling pathways responsible for CCL2 induction or E-selectin and the following activation state of the endothelium indirectly regulates endothelial CCL2 production needs to be further analyzed. One possible reason for decreased monocyte-derived CCL2 levels in E-selectin deficient mice may be a lack of monocyte activation. A study reported that monocyte-HUVEC interactions resulted in increased expression of CCL2 and IL-8, suggesting that once leukocytes adhered to the endothelium they may be activated by events of the extravasation cascade resulting in increased production of these chemokines (26). CCL2 is also well-known to trigger firm adhesion of monocytes to the vascular endothelium (27). Our results demonstrate an important role for E-selectin as an initiator of appropriate endothelial activation processes during metastasis. Thereby CCL2 production and E-selectin expression are linked and possibly participate in a positive feedback loop potentiating an inflammatory milieu. We believe that endothelial activation and E-selectin are essential for myeloid cell-derived CCL2 secretion into the metastatic niche. For this reason we next investigated E-selectin-mediated interactions with leukocytes during metastasis.

4. E-selectin-mediated leukocyte adhesion facilitates metastasis

Leukocytes in the metastatic niche are considered to be important contributors during metastasis since many studies have shown that recruitment and activation of immune cells,

especially inflammatory monocytes, are strongly associated with enhanced metastatic colonization. Monocytes are either recruited by primary tumors to metastatic target sites or attracted by intravascular tumor cells. They are a critical source of various soluble factors such as growth factors, matrix degrading enzymes, cytokines and chemokines and have been reported to assist tumor cell survival in the microvasculature and transmigration through the endothelium (21,28-32). In our study, we showed that the recruitment of myeloid cells, including macrophages, inflammatory monocytes and granulocytes, to metastatic sites is E-selectin-dependent since less of these myeloid cell populations were found in the lungs of E-selectin deficient mice within the first 2 days after tumor cell injection. On one hand this could be a consequence of weaker endothelial cell activation and therefore reduced CCL2 levels. On the other hand, a lack of E-selectin and diminished CCL2 levels could prevent efficient capturing and firm adhesion of recruited monocytes. We demonstrated reduced tumor cell-leukocyte association at metastatic sites in E-selectin deficient mice, indicating that the local recruitment of leukocytes to tumor cells is E-selectin-dependent. Accordingly, flow adhesion experiments *in vitro* demonstrated less monocytes associating with tumor cells on E-selectin deficient endothelial monolayer. Based on these results, vascular E-selectin directly regulates the adhesion of leukocytes in the vicinity of tumor cells whereby leukocytes probably get activated leading to CCL2 secretion.

5. Interactions between E-selectin and ligands on monocytes induce vascular permeability and tumor cell transmigration

Previous studies have already revealed that selectin-mediated leukocyte interactions strongly support metastasis during early stages. Decreased leukocyte-tumor cell interactions and impaired early tumor cell seeding have been observed in L-selectin deficient mice (33). In mice unable to induce L-selectin ligand expression at sites of intravascular tumor cell arrest metastasis is attenuated (29). Mice lacking fucosyltransferase-4/7 have no endogenous selectin ligands on leukocytes and exhibit less recruitment of monocytes to the metastatic microenvironment and attenuated metastasis (32). This study demonstrates that metastasis

is promoted by monocytes interacting with the endothelium. The role of monocytes during metastasis has been elaborated recently whereby tumor cell-derived CCL2 signals via endothelial CCR2 and thereby promotes tumor cell extravasation (21). The JAK2-Stat5-p38 MAPK signaling pathway is activated in the CCR2 expressing endothelial cells via concerted actions of tumor-cell derived CCL2 and inflammatory monocytes, consequently increasing vascular permeability and driving tumor cell transmigration. Whether only tumor-cell secreted CCL2 or also monocyte or endothelial CCL2 contributes to the activation of the JAK2-Stat5-p38MAPK pathway is not known. Also the role of interactions between E-selectin on endothelial cells and its ligands on monocytes was not analyzed in this study but it's quite conceivable that they might participate in triggering these signaling events.

In our study we observed enhanced monocyte-assisted tumor cell transmigration *in vitro* in the presence of E-selectin on the endothelium which was diminished when E-selectin was missing on the endothelium. Non-viable (fixed) monocytes which display ligands, including those for E-selectin, on their surface as well as fucosyltransferase-7 deficient monocytes with a limited amount of selectin ligands were able to support tumor cell transmigration. Fixed fucosyltransferase-7 deficient monocytes didn't assist tumor cells to transmigrate. These observations show that E-selectin ligands on monocytes and soluble factors from viable monocytes are required for efficient tumor cell transmigration. It is well known that the interaction of ligands on leukocytes with endothelial E-selectin induces signaling pathways which initiate the extravasation cascade in endothelial cells via "outside-in" signaling. This has been demonstrated by many studies *in vitro* using HUVECs whereby E-selectin-ligand interactions lead to signaling in endothelial cells via MAPK, PLC-γ, Erk/Src and MLC/p38 (34,35). These signaling events promote increased monolayer permeability (36) due to disruption of VE-cadherin/β-catenin complexes or the formation of stress fibers which both facilitate trans-endothelial migration (37,38). Consistent with these findings, we demonstrate that monocytes assist tumor cells by inducing cytoskeletal retraction in endothelial cells by interacting with E-selectin. This effect was diminished in the same experimental set up using E-selectin deficient endothelial cells. Our results indicated that E-selectin on endothelial cells

constitutes an important initiator of the extravasation cascade via its interaction with ligands on monocytes, resulting in signal transduction in endothelial cells as well as in monocytes which manipulates both cell types in a way that promotes tumor cell extravasation.

We discovered that induction of vascular permeability requires E-selectin *in vivo* since we observed almost no permeability changes in the lungs of E-selectin deficient mice upon tumor cell injection. We obtained similar results when we depleted monocytes prior to tumor cell injection indicating that endothelial E-selectin and monocytes are necessary for induction of pulmonary vascular permeability in response to tumor cells. These findings support the notion that E-selectin-mediated leukocyte adhesion and the resulting signaling cascade in the endothelium and leukocytes crucially modulate vascular integrity during metastasis.

6. E-selectin involvement during later stages of metastasis

Our results demonstrate that E-selectin also regulates the incidence of spontaneous metastasis. We demonstrated this in tumor bearing E-selectin deficient mice that had decreased lung metastasis. We therefore conclude that E-selectin plays a further role during “natural” spreading of primary tumors. It was reported that primary tumor factors can up-regulate E-selectin and the focal adhesion kinase to form hyperpermeable foci where tumor cells can extravasate (39). Further evidence is required to determine whether E-selectin regulates spontaneous metastasis through the same mechanisms we investigated with our experimental metastasis model or whether E-selectin has additional effects during metastasis development.

There is evidence that E-selectin is involved during essential events of angiogenesis as several studies have linked E-selectin with endothelial proliferation, (40,41) migration (42), and tube formation (43). Down-regulation of E-selectin resulted in diminished endothelial progenitor cell homing, tumor angiogenesis and tumor growth in transplantation models of human melanoma xenografts into mice (44). However the role of E-selectin during spontaneous metastasis, especially regarding growth of secondary tumors requires further evaluation. In addition, it would be of great interest to elucidate the interplay between cells of

the adaptive immune system and E-selectin expressing endothelial cells during early and late stages of metastasis. Some preliminary results of ours show attenuated experimental lung metastasis in Rag1 deficient mice (lacking mature lymphocytes) compared to C57BL/6 mice. This effect is even greater in Rag1/E-selectin double deficient mice.

7. Conclusion and outlook

This thesis describes a novel mechanism whereby vascular E-selectin assists tumor cells during extravasation by interacting with leukocytes at the metastatic site. Based on our results we propose the course of events illustrated in Figure 1: Tumor cells undergo intravascular arrest and produce CCL2 which activates endothelial cells which in turn up-regulate endothelial E-selectin expression. On one hand this triggers further CCL2 production by endothelial cells and on the other hand inflammatory monocytes are recruited to the metastatic sites in response to tumor- and endothelial cell-derived CCL2 and other factors. Recruited monocytes bind to E-selectin via surface ligands which enables their adhesion to the microvasculature and triggers signaling events both in endothelial cells and monocytes. In activated monocytes CCL2 production is further amplified while E-selectin-ligand interactions activate signaling pathways that lead to cytoskeletal rearrangement and consequently retraction of endothelial cells. Besides, CCL2 in the metastatic niche also enhances the firm adhesion of monocytes (mediated by integrin-VCAM-1/ICAM-1 interactions) and contributes to breaching of the vascular barrier. Finally, tumor cells extravasate through local vascular openings and colonize the lung parenchyma (Figure 1).

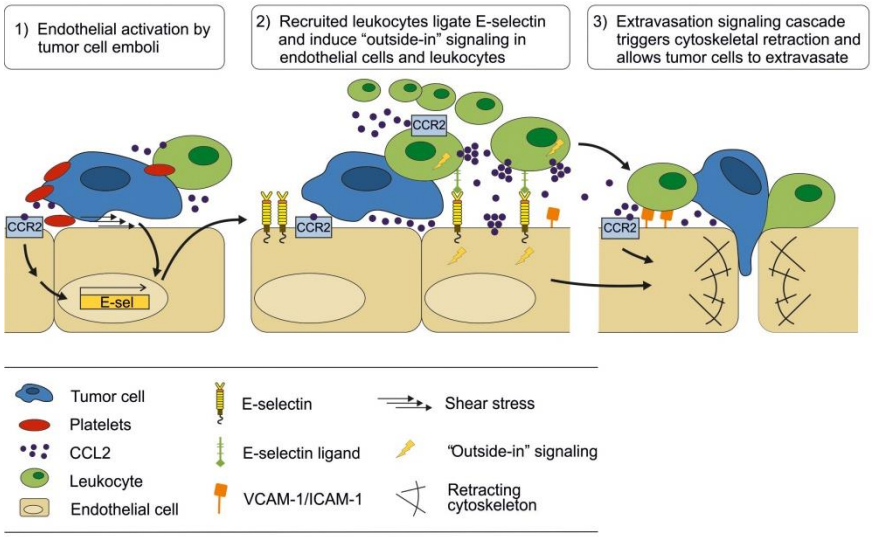

Figure 1. E-selectin mediated interactions with leukocytes facilitate tumor cell transmigration.

Thus, E-selectin-mediated interactions between the endothelium and leukocytes in combination with CCL2-mediated signaling during the metastatic process play a critical role as "architects" of the metastatic niche by enabling tumor cell extravasation and initiating the successful development of secondary tumors. Understanding mechanisms driven by E-selectin-leukocyte interactions leading to metastasis will help to improve our understanding of the complex processes taking place during tumor spreading and offer new prospects for future clinical applications.

Our findings gave rise to several additional questions:

- What are the signaling pathways that are responsible for the tumor cell-derived CCL2-mediated up-regulation of E-selectin in the vasculature?
- How does E-selectin in turn trigger CCL2 expression by endothelial cells?
- How does the endothelial E-selectin-leukocyte ligand interaction induce CCL2 expression by leukocytes?
- Can E-selectin-leukocyte ligand interactions be pharmacologically impeded to prevent metastasis formation?

Hence, continuing investigations will provide more mechanistic details and might help to therapeutically target specific interactions involving endothelial E-selectin during metastasis.

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APENDIX

Review: Altered tumor-cell glycosylation promotes metastasis

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Altered tumor-cell glycosylation promotes metastasis

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Malignant transformation of cells is associated with aberrant glycosylation presented on the cell-surface. Commonly observed changes in glycan structures during malignancy encompass aberrant expression and glycosylation of mucins; abnormal branching of *N*-glycans; and increased presence of sialic acid on proteins and glycolipids. Accumulating evidence supports the notion that the presence of certain glycan structures correlates with cancer progression by affecting tumor-cell invasiveness, ability to disseminate through the blood circulation and to metastasize in distant organs. During metastasis tumor-cell-derived glycans enable binding to cells in their microenvironment including endothelium and blood constituents through glycan-binding receptors – lectins. In this review, we will discuss current concepts how tumor-cell-derived glycans contribute to metastasis with the focus on three types of lectins: siglecs, galectins, and selectins. Siglecs are present on virtually all hematopoietic cells and usually negatively regulate immune responses. Galectins are mostly expressed by tumor cells and support tumor-cell survival. Selectins are vascular adhesion receptors that promote tumor-cell dissemination. All lectins facilitate interactions within the tumor microenvironment and thereby promote cancer progression. The identification of mechanisms how tumor glycans contribute to metastasis may help to improve diagnosis, prognosis, and aid to develop clinical strategies to prevent metastasis.

Keywords: glycosylation, cancer, metastasis, glycan ligands, mucins, siglecs, galectins, selectins

INTRODUCTION

The majority of cancer deaths are attributed to the metastatic spread of cancer cells to vital organs rather than to the primary tumor outgrowth. During malignant transformation, the genetic alteration in the cells results in mutations of proto-oncogenes and tumor suppressor genes, which as a result give rise to tumor clones with different properties (1). Malignant cells thereby acquire characteristics enabling them dissociation from tumors, degradation of the extracellular matrix, invasion, adhesion, and metastasis to distant organs. Alteration of tumor-cell-surface glycosylation is one of the characteristic traits associated with enhanced malignancy (2–4). Glycans are oligosaccharide structures that are covalently bound to proteins, lipids, or present in a free form in tissues or tumors. Glycans are bound to the protein either through Asn (*N*-linked glycan) or through Ser or Thr (*O*-linked glycan). Lectins are a family of carbohydrate-binding proteins that specifically recognize glycans. Fundamental processes such as cell–cell recognition, cell adhesion, mobility, and pathogen–host interaction are facilitated by lectins in healthy organisms. The common expression of lectins on endothelial cells, immune cells, in the extracellular matrix or as soluble adhesion molecules enables them to bind to tumor-cell glycans and thereby affect tumor-cell progression (5). Subsequently, accumulating evidence supports the involvement of tumor-cell-surface glycans in tumor-cell migration, adhesion, and metastasis. This review addresses the role of cancer-associated glycans during metastasis with the focus on endogenous lectin interactions within the tumor microenvironment.

THE PROCESS OF METASTASIS

Hematogenous metastasis is a multistep process during which malignant cells detach from the primary tumors, degrade the extracellular matrix, invade the surrounding tissue, enter the blood or lymphatic vessels, and extravasate to form metastatic lesions. Tumor cells through the cell-surface glycans can engage with a variety of endogenous lectins both at the primary site of a tumor and in the circulation. Tumor cell upon reaching the blood circulation induces microthrombi, the formation of which is facilitated by platelet P-selectin binding to tumor-cell-surface glycans (6, 7). Tumor-cell emboli formation contributes to mechanical lodging in the microvasculature and/or adhesion to the endothelium thereby promoting tumor-cell extravasation and metastasis (8). There is accumulating evidence that vascular lectins–selectins facilitate tumor-cell interactions with all blood constituents, platelets, leukocytes, and endothelial cells, and thereby contribute to metastasis (3, 9, 10). In addition, recruitment of immune cells to the metastatic microenvironment is dependent on selectins (11–13).

Specific glycan structures on colonic epithelium provide immune-modulatory activity to tissue macrophages through sialic acid-binding lectins–siglecs (14, 15). In addition, galactose-binding lectins–galectins were shown to be involved in immune-suppression and metastasis (16). Consequently, altered glycosylation may both induce inflammatory reactions and promote immune-suppression, however; it is dependent on the cellular context within the tissue. Finally, glycan changes associated with cancer progression profoundly define the phenotype of cancer cells depending on interactions with endogenous lectins both in tumor and metastatic environments.

GENERAL MECHANISMS FOR ALTERED GLYCOSYLATION IN CANCER

Cancer progression requires a range of alterations in extracellular and intercellular signaling that promotes cell proliferation,

emergence of invasive subsets, dissociation from the tumor, intravasation, and adhesive interactions within the circulation that finally facilitate metastasis. Within the tumor environment changes in glycosylation allow malignant cells to promote cell mobility, cell adhesion, and even receptor activation, and thereby contributing to the invasive phenotype (3–5). Malignant transformation leads to expression of oncofetal antigens, epitopes that are present on embryonic tissues and tumor cells, but are generally absent in healthy adult cells. Neo-synthesis and incomplete synthesis are the two major mechanisms for generation of cancer-specific glycans (2).

Altered glycosylation of *N*-linked glycans in cancer is typically associated with enhanced β1,6-branching (**Table 1**) that is facilitated by β1,6-*N*-acetylglucosaminyltransferase-5 (GnT5) (17, 18). Increased activity of GnT5 is associated with increased polylactosaminic sequences, and the inhibition of GnT5 resulted in attenuation of metastasis (19, 20). GnT5 deficiency (Mgat5-deficient mice) resulted in reduced tumor growth and metastasis (21). However, the functional role of branched N-glycosylation in cancer was later shown to be dependent on galectin binding and thereby altering the phenotype of the cell (22).

Virtually in every cancer type upregulation of glycosyltransferases has been detected, leading to expression of common tumor-cell epitopes such as sialyl-Lewis^x and sialyl-Lewis^a (sLe^x/sLe^a), Thomsen-nouvelle antigen (Tn), and sialyl-Tn (sTn) (3–5, 23, 24). Hypoxia has been identified as one of the factors leading to increased expression of glycosyltransferases (25, 26). For instance, increased expression of α1,3-fucosyltransferase-7 (FUT7) and α2,3-sialyltransferase ST3Gal1, enzymes involved in synthesis of sLe$^{x/a}$ has been detected (27). The general increase in sialylation has been detected both in clinical settings and experimental models that is associated with a metastatic cell phenotype (25, 28, 29). An increase in α2,6-sialylation in tumors is usually attributed to the upregulation of ST6Gal1 sialyltransferase that is primarily active on *N*-linked glycans (30–32), or ST6GalNAc family of sialyltransferases, which are active on *O*-linked glycans or glycolipids (33). Accordingly, overexpression of Neu1 sialidase in colon cancer cells led to reduced liver metastasis in mice due to increased desialylation of β4 integrin whereas silencing of Neu1 sialidase increased cell migration, invasion, and adhesion *in vitro* (34).

Synthesis of shorter glycan structures like Thomsen–Friedenreich (TF or T), Tn, and sTn epitopes has been observed in a number of carcinomas (35–39). One of the factors affecting the synthesis of incomplete glycan structures is the frequent mutation of the *Cosmc* chaperone that is required for the galactosyltransferase activity that modifies *O*-linked glycans (40). Another example of shortened glycan synthesis is the reduced expression of disialyl-Lewis^a (di-sLe^a) and sialyl 6-sulfo Lewis^x structures in epithelial cancer. Disialyl-Lewis^x (di-sLe^x) structure is synthesized with the α2,6-sialyltransferase ST6GalNAc6, and its expression is downregulated by epigenetic silencing in malignant epithelium (41, 42). Similarly, repressed expression of sulfotransferase responsible for 6-sulfo Le^x was detected in cancer cells but not in normal epithelial cells (26).

Gangliosides are sialic acid-containing glycolipids, which expression is often dysregulated during malignant transformation

Table 1 | Common glycan alterations on carcinoma cells and their effect on lectin recognition.

Structural change	Carriers	Biosynthetic basis of structural change	Potential lectin partners	Reference
Increased β1,6-branching (*N*-linked)	*N*-glycans	Increased GnT5	Galectins Siglecs	Guo et al. (19), Lagana et al. (20)
Increased α2,6-sialylation	*N*-glycans, e.g., β integrin	Increased ST6Gal1 sialyltransferase		Seales et al. (32)
General increase in sialylation	Mucins *N*-glycans	Increased sialyltransferase activity	Selectins, siglecs, galectins	Dall'Olio et al. (30), Gessner et al. (31)
Increased sialyl-Lewis$^{x/a}$	Mucins	Increased FUT7, FUT3, FUT6, ST3Gal6	Selectins	Barthel et al. (169), Julien et al. (195), Koike et al. (27), Ogawa et al. (161), Yin et al. (198)
Decreased di-sialyl-Lewis$^{x/a}$	Mucins, glycolipids	Decreased ST6GalNAc6 GlcNAc6ST1	Selectins Reduced siglecs binding	Miyazaki et al. (41), Tsuchida et al. (42) Nudelman et al. (43)
Increased Tn epitopes	Mucins (e.g., MUC1), CD44, β1 integrin, osteopontin	Downregulated T-synthase activity due to Cosmc mutations	Galectins	Ju et al. (40)
Increased sialyl-Tn epitopes		Increased ST6GalNAc1 expression	Siglecs Galectins	Julien et al. (67), Ozaki et al. (68)
Increased T antigen (core 1 structure)		Decreased C2GnT2 Enhanced availability of UDP-galactose	Galectins	Brockhausen et al. (53), Dalziel et al. (55) Kumamoto et al. (73)
Increased sialyl-T antigens		Increased levels of α2,3-sialyltransferase (ST3Gal1)	Galectins Siglecs	Burchell et al. (78), Dalziel et al. (55), Picco et al. (79), Schneider et al. (72)

(2). Apart from glycolipid specific glycan structures containing disialic acid in a α2,8-linkage (e.g., GD3), changes in glycosyltransferases promote expression of sLe^x epitopes (43). Overexpression of sialidase Neu2 led to reduced metastasis, while Neu2 was found to be downregulated in highly metastatic variants of colon carcinoma (44).

Despite many possibilities for the formation of glycans (linkage and sequence of monosaccharide units) there is a rather small number of structures commonly detected in cancer. Furthermore, terminal glycan structures exposed on the cell surfaces of tumor cells can be recognized through endogenous lectins and thereby modulate cancer progression.

ALTERATIONS OF CANCER-ASSOCIATED *O*-LINKED GLYCANS

Mucins are high molecular weight glycoproteins exhibiting a rod like conformation due to heavy glycosylation with *O*-linked glycans (3, 45). *O*-linked glycosylation, which is based on GalNAc bound to the Ser/Thr of a protein, is further modified by galactose (core 1 structure) or GlcNAc (core 3 structure) in normal mucins (**Figure 1**). During malignant transformation mucins of intestine, colon, liver, and pancreas have reduced core 1 and core 3 structures that correlate with enhanced sialylation of Tn and T antigens (24, 46, 47). Core 3-derived glycans are a major type expressed by normal epithelial cells of the gastrointestinal tract, which are downregulated during malignancy due to loss of functional β3-*N*-acetylglucosaminyltransferase-6 (core 3 synthase) expression (48, 49). Consequently, overexpression of core 3 synthase in pancreatic cells was associated with decreased presence of Tn antigens and resulted in a reduced tumorigenicity and metastasis upon orthotopic injection. In addition, enhanced expression of the core 2 β1,6-*N*-acetylglucosaminyltransferase (C2GnT1) responsible for the core 2 synthesis was detected in colorectal and lung carcinomas, which correlated with high levels of sLe^x on *O*-glycans and therefore strong binding to E-selectin and metastasis compared to normal tissues (50–52). Mucins of normal mammary epithelial cells contain a mixture of *O*-glycans and the majority is core 2-based structures (53, 54). Reduced expression of C2GnT1 in mammary cancer is associated with enhanced presence of Tn and sTn (53–56). However, despite reduced core 2 structures on breast cancer cells, increased presence of sLe^x epitopes has been observed, which likely is a result of increased fucosylation (57).

FORMATION OF T, Tn, AND sTn ANTIGENS DURING CANCER PROGRESSION

In healthy tissues, core 1-based T and Tn epitopes are almost absent however; in about 90% of all human carcinomas these precursor structures are detected (36, 39). Unsubstituted Tn epitopes occur in human cancers of colon, breast, bladder, prostate, liver, ovary, and stomach; and their presence correlate with cancer progression and metastasis (35–37, 58–63). Similarly, sialylated T and Tn antigens correlate with progression of epithelial cancer and poor clinical prognosis of many carcinomas (25, 28, 39, 64–66). ST6GalNAc1-mediated α2,6-linked sialylation of GalNAc of the precursor Tn antigen results in formation of the sTn antigen (25, 67–69). The sialylation step prevents further glycan extension and therefore leads to truncation of *O*-linked glycans (47, 70).

Several mechanisms have been described to enable increased Tn, sTn, or T expression in cancer (**Table 1**) (33, 46). (1) Decreased activity of core 2 C2GnT1 enzyme leads to accumulation of T antigen (described above) that is further sialylated by ST6GalNAc1

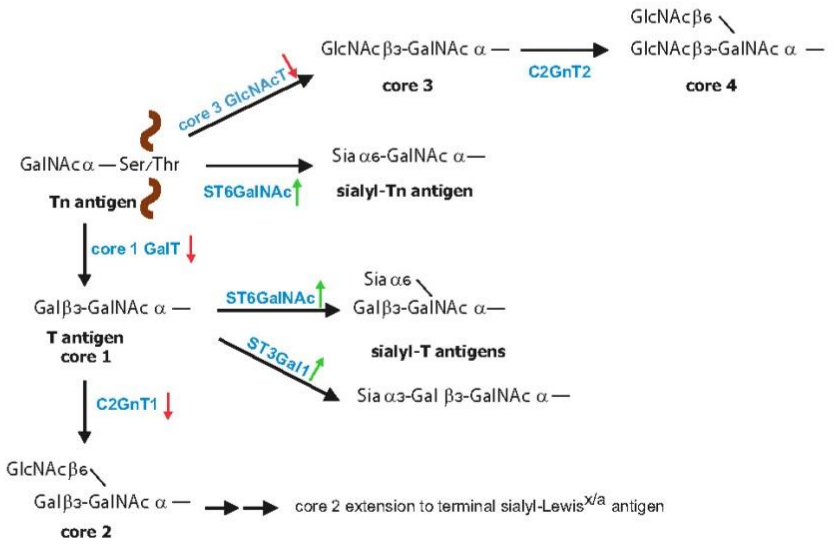

FIGURE 1 | Biosynthesis of *O*-glycans. *O*-glycan synthesis is initiated by linking of GalNAc to the protein at Ser or Thr residue. The simplest *O*-glycan Tn antigen can be further converted to core 1 structure (T antigen) by β1,3 galactose extension; core 3 structure by addition of β1,3-GlcNAc. During cancer increased expression (green arrow) of sialyltransferases with concomitant reduced expression (red arrow) of core 1 GalT and core 3 GlcNAcT leads to increased formation of sialyl-Tn and sialyl-T antigens. Core 1 structure is further branched by C2GnT1 to form core 2 that can be further modified to poly-*N*-acetyllactosamine structures carrying sialyl-Lewis$^{x/a}$ [modified from Ref. (23)].

and ST6GalNAc2 enzymes (71, 72). (2) Enhanced availability of the nucleotide sugar substrate UDP-galactose appears to promote increased T antigen biosynthesis through core 1 β1,3-galactosyltransferase (73). Colon cancer tissues expressed increased levels of the UDP-Galactose transporter, which brings the sugar donor into the Golgi apparatus compared to non-malignant mucosa. (3) Activity of β1,3-galactosyltransferase (T synthase) requires the presence of the molecular chaperon protein *Cosmc*, which is responsible for folding and stability of the enzyme (40, 74). The absence of *Cosmc* leads to β1,3-galactosyltransferase degradation. Mutation in *Cosmc* chaperone is associated with increased Tn expression in colon carcinoma and melanoma cell lines and also increased sTn expression (40, 75). Accordingly, down-regulation of T-synthase resulted in a marked increase of T, Tn, and particularly sTn in colon carcinoma cells (76). (4) Generation of sTn is facilitated by the sialyltransferase ST6GalNAc1 and ST6GalNAc2 (71, 72). Human gastric cancer cells with enhanced ST6GalNAc1 expression showed higher intraperitoneal metastasis compared to sTn-negative tumor cells. Similarly, overexpression of ST6GalNAc1, thereby sTn epitope, in human breast cancer cells led to increased tumor growth in immunodeficient mice (68, 77). In addition, enhanced sialylation of T antigen in breast cancer correlated with higher levels of α2,3-sialyltransferase (ST3Gal1) (72, 78). Overexpression of ST3Gal1 under the human MUC1 promoter in a spontaneous murine breast cancer model resulted in significantly decreased tumor latency compared to mice without ST3Gal1 overexpression (79). Furthermore, the sialyltransferase expression alone was responsible for enhanced tumorigenesis indicating that this enzyme *per se* acts as a tumor promoter (79).

Only few glycoproteins are known to present Tn, T, or sTn and sialyl-T (sT) antigens in malignant tissues (66). Mucin MUC1 and CD44v6 display sTn and sT antigens in colon, gastric, and breast cancers (80–83). MUC2 is a major carrier of shortened glycans in gastric cancer (84). Enhanced sTn expression in breast and gastric cancer is associated with overexpression of MUC1, CD44, and ST6GalNAc1 (68, 77). Although CD44v6 is expressed in some types of healthy epithelia, higher expression is observed in squamous cell carcinomas and adenocarcinomas including breast, lung, colon, and pancreatic carcinomas (85–87). Interestingly, serum levels of osteopontin, a CD44 ligand, that itself is a sTn carrier, have been detected in cancer patients and correlate with poor prognosis (87).

The enhanced expression of Tn, sTn, and T antigens on MUC1, osteopontin, and CD44 is associated with high metastatic potential and poor prognosis (84, 88, 89). However, there is little evidence for the functional consequence of this aberrant glycosylation during cancer progression. In human breast cancer cells, expression of sTn on MUC1 was associated with reduced cell adhesion and increased cell migration (77). In addition, β1 integrins carry aberrant forms of *O*-glycans that is associated with metastasis (90). Enhanced expression of ST6GalNAc1 in murine carcinoma cells led to an increase in sTn expression on β1 integrin subunit associated with morphological changes including loss of epithelial appearance, disorganization of actin stress fibers, and reduced ability to migrate on fibronectin. A recent study showed that high expression of the ppGalNAcT13, which initiates *O*-glycan synthesis by adding the first GalNAc to Ser/Thr, induced high metastatic potential of Lewis lung carcinoma by generating trimeric Tn antigens $(GalNAc1\text{-}Ser/Thr)_3$ on syndecan 1 (91). The complex formation of trimeric Tn antigens on Syndecan 1 together with α5β1 integrin and MMP-9 resulted in enhanced invasion and metastasis. Recent findings provide evidence that cell-surface mucins are involved in signal transduction events [reviewed in Ref. (24, 45)]. Decreased sTn expression on neuroblastoma achieved by extension of core 1 structure with B3GNT3 expression reduced activation of focal adhesion kinase and thereby partially suppressed malignant phenotype (92). Aberrant glycosylation in cancer does not affect only the tumor-cell phenotype behavior (e.g., proliferation, differentiation, and adhesion), but also contribute to the control of the local microenvironment, immune responses, and metastasis. Therefore, these glycans serve as ligands for cells in the tumor microenvironment through endogenous lectins.

SIGLECS

Sialic acid-binding immunoglobulin superfamily lectins (siglecs) are the largest family of sialic-acid-binding molecules (93–95). Siglecs are expressed on specific subpopulations of hematopoietic cells where they exert their immune-regulatory function. Many siglecs contain intracellular tyrosine motifs, which include one or more membrane-proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) and a membrane-distal ITIM-like motif (93, 94). These motifs are involved in inhibitory signal transduction. Based on both sequence similarity and conservation between mammalian species siglecs are divided in two major subgroups. The first group comprises Siglec-1 (sialoadhesin, CD169), Siglec-2 (CD22), Siglec-4 (myeloid-associated glycoprotein), and Siglec-15. The second subfamily of CD33/Siglec-3 related siglecs consists of 10 human members (Siglec-3, -5, -6, -7, -8, -9, -10, -11, -14, and -16) and 5 rodent members (Siglec-3, -E, -F, -G, and -H) (93, 95). The first subgroup with its evolutionary conserved members has restricted expression patterns. For instance Siglec-1 is specifically expressed on macrophages, Siglec-2 on B-cells and Siglec-4 on oligodendrocytes and Schwann cells in the nervous system (96). On the other hand, CD33-related siglecs display a more divergent expression pattern dependent on developmental stage of immune cells (93, 95). The high sialic acid concentration on the cell-surface of siglec-expressing cells often leads to binding to the cell glycans (in cis) or adjacent cells (in trans). Siglecs can be affected by various stimuli including cytokines, toll-like receptor activation, and viral and bacterial infections, the biology of siglecs is therefore rather complex (96). The binding specificity of siglecs depends on the distinct types, linkages (α2,3, α2,6, and α2,8), arrangements of sialic acids, their way of presentation on different cells, organs, and organisms. Siglec binding to ligands modulates cell–cell interactions, cell proliferation, cell death, and endocytosis (96–99).

THE ROLE OF SIGLECS IN CANCER PROGRESSION

Accumulating evidence indicates that the interaction between tumor-specific glycans and lectins on immune cells are involved in modulation of the tumor microenvironment (100). The inhibitory nature of siglec upon binding of specific glycan may lead to dampening of immune responses and thereby escape of immune surveillance and clearance. Whether siglecs contribute to cancer

progression through recognition of distinct cancer-specific glycan structures is currently under investigation. Non-malignant colon epithelial cells express di-sLe^a epitopes that serve as ligands for both Siglec-7 and -9 (15). The expression of siglec ligands was decreased upon malignant transformation, which was associated with enhanced expression of sLe^x and sLe^a epitopes (26). Expression of ST6GalNAc6, which synthesizes di-sLe^a in human colon cancer cells resulted in increased di-sLe^a, loss of sLe^a epitopes, and increased binding to Siglec-7 (41). Mainly resident macrophages were found to carry Siglec-7 and -9 in a colonic lamina propria and Siglec-7/9 ligation could suppress macrophage-mediated cyclooxygenase-2 (COX2) and prostaglandin E2 expression and thereby prevent inflammatory damage of the colonic mucosa (15). Siglec-15, which preferentially recognizes sTn antigen, is expressed in tumor-associated macrophages (TAMs) in various human carcinoma tissues including lung, liver, and rectum (101). Binding of myeloid cells through Siglec-15 to sTn on tumor cells resulted in increased TGF-β secretion into the tumor microenvironment that is associated with cancer progression. Interestingly, Siglec-15 expression was induced by M-CSF, which usually polarizes macrophages to M2 phenotype commonly detected in the tumor microenvironment.

Siglec-1 is expressed in a subset of macrophages that are involved in the pathophysiology of cancer (102). Clinical observation showed that increased Siglec-1 is present in splenic marginal cell lymphoma as well as in macrophage infiltrates of MUC1-positive breast cancers (103, 104). Siglec-1 positive macrophages were found to infiltrate into rat xenograft tumors in a CCL2-dependent manner (105). On contrary, recent study demonstrated that Siglec-1 positive macrophages in regional lymph nodes of colorectal carcinoma patients promote CD8$^+$ T-cell mediated anti-tumor immunity and are associated with a better prognosis for these patients (106).

Siglec-9, a surface receptor on NK cells, B-cells, and monocytes, has been identified as a receptor for mucin MUC16 (14). Cell-surface bound as well as soluble MUC16 is overexpressed in human ovarian tumor cells and detected in peritoneal fluid of cancer patients (107). Engagement of Siglec-9 on monocytes also induced secretion of immunosuppressive cytokine IL-10 (108). Similar immune-suppression mediated by Siglec-7 on NK cells was observed in renal cell carcinoma expressing disialosyl globopentaosylceramide (DSGb5) as a major ganglioside (109). Recent study from C. Bertozzi group provided strong evidence that siglec-7-mediated cytotoxicity of NK cells can be modulated by the alteration of glycans on cell surfaces (110). Presentation of sialylated ligands on tumor cells recognized by siglec-7 resulted in enhanced phosphorylation of cytoplasmic tyrosine residues, causing dampening of cytolytic activity.

The association between Siglec-9 positive immune cells and MUC1-positive tumor cells has been detected in tissues of human colon, pancreas, and breast cancer. Interestingly, Siglec-9 binding to MUC1 expressing tumor cells was shown to induce recruitment of β-catenin in tumor cells resulting in promotion of cell growth *in vitro* (111). These findings suggest that Siglec-9 engagement of carcinoma mucin MUC1 may be involved in tumor growth, however; the nature of Siglec-9 ligands as well as the cellular context *in vivo* remains to be defined.

Taken together, the current evidence is largely based on clinical correlation of cancer–glycan expression and several experiments showing Siglec-cancer–glycan interaction *in vitro*. Whether these interactions indeed functionally modulate immune cell responses in the tumor microenvironment and thereby affect cancer progression *in vivo* requires experimental validation.

SIGLECS AS TARGET OF CANCER THERAPY

The identification of Siglec-2 and Siglec-3 as markers of acute myeloid leukemia (AML) and B-cell lymphomas raised interest in potential immunotherapy (112–114). Anti-Siglec-2 and siglec-3 specific antibodies were conjugated with variety of toxins and such immunotoxins have been targeted in several autoimmune diseases and hematological malignancies [reviewed in Ref. (93, 94, 115)]. In the majority of acute lymphoblastic leukemias (ALL) Siglec-2 (CD22) was identified as a useful target for cell-depletion therapy (116). Inotuzumab ozogamicin is an immunotoxin comprised of a humanized IgG4 monoclonal antibody covalently linked to calecheamicin (CMC-544). CMC-544 was active against B-cell tumors in preclinical models and has been evaluated in phase I study for patients with B-cell lineage ALL (117). Inotuzumab ozogamicin used as a single therapy in patients with refractory-relapsed ALL showed positive results.

The immunotoxin gemetuzumab ozogamicin (OG, Mylotarg; Wyeth, Madison, NJ, USA), which consists of a humanized anti-CD33 (siglec-3) murine antibody linked to calicheamicin, was approved by the FDA for treatment of CD33+ AML patients. Binding and endocytosis of the conjugate resulted in the intracellular release of the toxin causing cell death of CD33+ cells (94, 115). However the drug is off the market since 2010 because the key phase III trial (South West Oncology Group Study S0106) in which GO was combined with induction chemotherapy failed to improve disease-free survival and caused higher fatal induction toxicity rate compared to chemotherapy alone (118). Recent studies using lower or fractionated dose of GO suggest that GO may still improve survival of distinct subsets of AML patients, particularly patients with favorable cytogenetics (119). New approaches with humanized CD33 antibody conjugated to synthetic DNA cross-linking pyrrolobenzodiazepine (SGN-CD33A) have been developed and revealed promising effectiveness in animal models (120). SGN-CD33A is now currently being tested in a phase I trial (ClinicalTrials.gov: NCT01902329).

GALECTINS

In contrast to siglecs and selectins, which are mostly cell-surface-bound receptors, galectins are soluble immunomodulatory lectins (121). Galectins bind to galactose that is either β1,3- or β1,4-linked to *N*-acetylglucosamine, a common disaccharide found both on *N*- and *O*-linked glycans and glycolipids. Galectins act both intracellularly by modulating signaling pathways and extracellularly as regulatory receptors (100). Up to date the galectin family consists of 15 members, which are classified into three groups based on structural differences: prototype galectins (Galectin-1, -2, -5, -7, -10, -11, -13, -14, and -15) having one carbohydrate recognition domain (CRD), tandem repeat-type galectins (Galectin-4, -6, -8, -9, and -12) having two CRDs, and the single member Galectin-3,

which has one CRD connected to a non-lectin N-terminal region responsible for oligomerization (100). Galectins are expressed by various cell types including epithelial and immune cells, but their expression is altered during progression of colon, breast, lung, pancreatic, head and neck, and cervical cancers (16, 122). Many studies indicate that cancer-associated galectins could regulate cancer cell proliferation, signaling, adhesion, invasion, and metastasis (122–124). Galectin-1 and Galectin-3 were most intensively studied in context of cancer.

GALECTIN-1

Accumulating evidence indicate that tumor-derived Galectin-1 contributes to immunosuppressive activity in different tumors, including lung and pancreatic carcinoma, melanoma, and neuroblastoma (16, 125–127). It has been shown that Galectin-1 binding to T-cells through *N*- and *O*-linked glycans on CD43 or CD45 mucins induces apoptosis of activated T-cells (128, 129). Galectin-1 expression by melanoma cells induced apoptosis of tumor-specific effector T-cells, and Galectin-1 inhibition allowed generation of a tumor-specific T1 response (126). Modification of cell-surface glycosylation affects glycan pattern on T-cells and thereby changes Galectin-1 binding. Enhanced expression of α2,6-sialyltransferase-1 (ST6Gal1) selectively modified *N*-glycans on CD45 and thereby inhibited Galectin-1 binding (130). How Galectin-1 contributes to immune-suppression in tumors has been delineated in lung cancer (131). High expression of Galectin-1 in lung cancer cell lines, as well as in human tumor tissues, alters the phenotype of monocyte-derived dendritic cells and impairs T-cell response, concomitant with increased presence of regulatory T-cells (Tregs). The regulatory effect of Galectin-1 is mediated by increased expression of IL-10 in monocytes thereby inducing a Th2-dominant cytokine profile. The enhanced infiltration of $CD11c^+$ dendritic cells in human lung cancer samples has been recapitulated in a mouse model, which was completely omitted after transplantation of Galectin-1 silenced tumor cells. In another study, the amount of Galectin-1 positive cells correlated with the tumor grade in human breast cancer (132). Silencing of Galectin-1 in a metastatic murine mammary tumor led to a reduction of tumor growth and lung metastasis with a concomitant reduction in infiltrating regulatory T-cells.

Experimental evidence also suggests that Galectin-1 expressed on various tumor-cell types including hepatocellular carcinoma, melanoma, ovarian, and prostate cancer cells mediates tumor-cell adhesion to the extracellular matrix (133, 134). In addition, Galectin-1 mediated attachment of cancer cells to the extracellular matrix and endothelial cells through binding to CD44 and CD326 on murine breast and colon cancer cells (16). Galectin-1 might also be involved in formation of platelet-cancer cell complexes since it was shown to activate platelets (135). Murine breast, colon, and Lewis lung cancer cells with silenced Galectin-1 showed decreased lung metastasis, which was associated with increased T-cell numbers and reduced angiogenesis (16, 125). Taken together, tumor-derived Galectin-1 exerts its immunosuppressive function through binding to endogenous (non-tumor-derived) glycans and thereby contributes to cancer progression.

GALECTIN-3

There is accumulating evidence that the cancer-associated T, Tn, and sTn structures promote metastasis through binding to Galectin-3. Galectin-3 expression is also increased in patient sera of several cancer types and associated with increased risk of metastasis (136, 137). For instance, T antigen expression by breast and prostate cancer cells facilitated interactions with cancer-associated Galectin-3 or with endothelial associated Galectin-3 (66, 138–140). These interactions lead to homotypic aggregation of cancer cells, which protects cancer cells from apoptosis induced by the lack of adhesion to the extracellular matrix (139). In addition, cancer cell-associated T antigens can induce Galectin-3 expression on the endothelium, which enabled cancer-endothelium adhesion (140). Another study has shown that lysosomal-associated membrane protein-1 (LAMP-1) on highly metastatic melanoma cells carries *N*-acetyllactosaminyl structures, which are recognized by Galectin-3 on lung endothelial cells suggesting that lung endothelial galectin-3 can serve as anchor for LAMP-1 expressing tumor cells in the circulation (141).

A characteristic feature of galectins is the induction of complex formation by cross-linking glycoproteins, which can form multimers "lattice" microdomain (121). Complex *N*-glycans are formed by GnT5 modification of *N*-glycans that are the ligands for Galectin-3 (142). Expression of GnT5 has long been implicated in tumor progression and metastasis (17). In particular, the absence of GnT5 delayed tumor formation and suppressed metastasis (21). Accordingly, up-regulated GnT5 expression has been observed in various human cancers (18, 143); and the ectopic expression of the GnT-V in multiple epithelial cells resulted in increased cell motility, tumor formation, and enhanced metastasis (144, 145). Furthermore, GnT5-dependent modifications of tyrosine kinase receptors such as EGF, TGF-β, IGFR, and PDGF enhanced affinity to galectin-3 and thereby prolonged their cell-surface expression (22, 146). Galectin-3-induced lattice formation prevented the surface clearance of receptors by clathrin-dependent endocytosis and enabled interaction with inhibitory caveolin-1 domains.

Branched *O*-glycans with poly-*N*-acetyllactosamine structures are recognized by Galectin-3 (147). In C2GnT1-expressing bladder tumor cells core 2 *O*-glycans present on MHC class I-related chain A are bound to Galectin-3 that reduced the affinity for the activating NK cell receptors NKG2D, thereby impairing NK cell function and anti-tumor activity.

Recent findings suggest that Galectin-3 also regulates dynamics of N-cadherin and the lipid raft marker ganglioside GM1 (148). Accumulation of N-cadherin and GM1 at cell–cell junctions destabilized cell–cell junctions and thereby promoted tumor-cell migration. *N*-glycans on α5β1 integrin are important for their proper binding to fibronectin (149, 150). Increased GnT5 mediated β1,6-branching reduces cell-surface clustering of α5β1 integrin, specifically of the β1 subunit, resulting in a less adhesive phenotype due to reduced adhesion to fibronectin and modulates fibronectin matrix remodeling in tumors (20, 151). Thus, Galectin-3 lattice formation provides another mechanism how altered glycosylation contributes to the malignant and invasive phenotype of tumor cells (148).

SELECTINS

Selectins are vascular cell adhesion molecules that belong to a family of C-type lectins, which facilitate the initial attachment of leukocytes to the endothelium during the process of leukocyte extravasation. The selectin family consists of L-, E-, and P-selectin, which share around 50% sequence homology in their C-type lectin domain (152). L-selectin (LECAM-1 and CD62L) is constitutively expressed on almost all hematopoietic cell types including myeloid cells, naïve, and some activated memory T-cells (152) and enables adhesion of leukocytes to the activated endothelium or in high endothelial venules of the peripheral lymph nodes (153, 154). E-selectin (ELAM-1 and CD62E) is exclusively displayed on endothelial cells, which requires *de novo* expression in response to inflammatory stimuli such as TNF-α and Il-1β. However, skin and parts of the bone marrow microvasculature have been shown to constitutively express certain E-selectin levels (155). On contrary, P-selectin (PADGEM and CD62P) is stored in alpha-granules of platelets as well as in Weibel–Pallade bodies of endothelial cells and can be rapidly mobilized to the cell-surface upon activation of platelets or the endothelia. E- and P-selectin bind to ligands on myeloid cells (156), certain types of lymphocytes (152) but also to several types of tumor cells (157–159). Selectins are the most-studied lectins in cancer biology, which promote cell–cell interaction with tumor cells and their microenvironment (9). All three selectins have been shown to contribute to tumor dissemination and specifically facilitate processes when the tumor cells are in the circulation.

SELECTIN LIGAND EXPRESSION CORRELATES WITH CANCER PROGRESSION

There is compelling clinical and experimental evidence that overexpression of tetrasaccharides sLe^x and sLe^a correlates with poor prognosis due to enhanced metastatic phenotype in a number of cancer types, including colon, gastric, prostate, renal, pancreatic, and lung cancer (89, 160–165). Enhanced expression of $sLe^{x/a}$ on cancer cells correlated with increased ability to adhere to E-selectin or to the activated endothelial cells and stromal cells *in vitro* (157, 166–168). Furthermore, high cell-surface expression levels of sLe^x were linked to enhanced metastatic activity in various experimental metastasis models using human carcinoma cells compared to lower or minimal sLe^x expression (169–171).

The minimal recognition motif for all three selectins are tetrasaccharides $sLe^{x/a}$ (**Figure 2**) (172). SLe^x are terminal structures of *N*-or *O*-linked glycans attached to glycoproteins and glycolipids displayed by most circulating leukocytes and endothelial cells whereas sLe^a is detected on some epithelial cells but mostly on various tumor cells (3, 4, 173). The four glycosyltransferases *N*-acetylglucosaminyltransferase, β1,4-galactosyltransferase, α2,3-sialyltransferase, and α1,3-fucosyltransferase-7 are responsible for synthesis of sialyl-$Lewis^{a/x}$ structures on cells of the hematopoietic system (172, 174). Efficient selectin binding to carbohydrates usually requires a glycoprotein scaffold that facilitates the presentation of selectin ligands in clusters (175). One of the best characterized ligands for all three selectins is the P-selectin glycoprotein ligand-1 (PSGL-1), which is concentrated on the tips of microvilli on leukocyte surface (176). To the most common mucins carrying selectin ligands that are associated with cancer progression belong

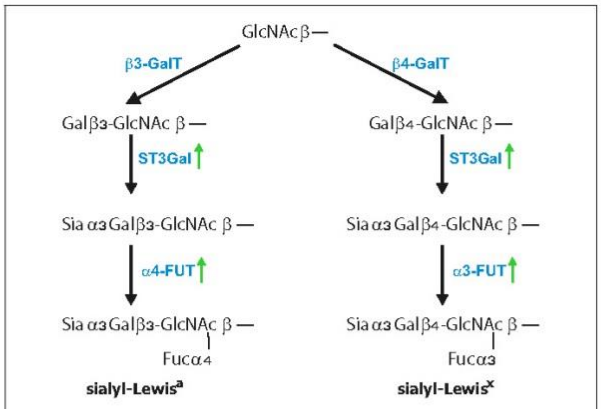

FIGURE 2 | Formation of Lewis antigens. Terminal GlcNAc residues, particularly on core 2 structures, are further extended by addition of β1,4 galactose, for $Lewis^x$ epitope, and β1,3 galactose, for $Lewis^a$ epitope. This is further followed by the addition of α2,3-linked sialic acid to Gal by ST3Gal enzymes and finalized by the addition of α1,3-linked fucose for sLe^x and α1,4-linked fucose for the sLe^a antigen. FUT3 finalized the synthesis of Le^a antigen, while FUT6 and FUT7 were shown to finalize Le^x epitopes.

MUC1, MUC2, MUC4, and MUC16 (35, 45, 177, 178). Apart from mucins, several other selectin ligand carriers on tumor cells have been identified that includes CD24, CD44, death-receptor 3, E-selectin ligand-1, PSGL-1, and podocalyxin-like protein and this list is by far not complete (179–183). Several of these ligands are also expressed on tumor cells and are associated with cancer progression. For instance, CD44 glycoproteins exist in several isoforms and are expressed on epithelial and endothelial cells as well as on multiple cancer cell types such as gastric, colorectal, pancreatic, and lung cancer (184–186). The aberrant expression of CD44 in colorectal carcinoma cells correlated with increased metastatic potential *in vivo* (187, 188). Based on flow-based adhesion assays *in vitro*, CD44v on human colon carcinoma cells binds to P-, E-, and L-selectin (189, 190). The majority of selectin ligands are presented on mucins, but they can be found equally functional also on *N*-linked glycans or glycolipids. Finally, P- and L-selectins also bind to heparin, heparan sulfate, and sulfated glycolipids, which also indicates certain flexibility in ligand recognition (9, 175). In addition, chondroitin sulfate glycosaminoglycans (CS-GAGs) on breast cancer cells were identified to serve as a P-selectin ligand that is associated with breast cancer metastasis (191). Despite the large variety of glycans, tumor cells express sialylated and fucosylated molecules, mostly on mucins which are also recognized by selectins (158, 159, 167, 192, 193).

Increased expression of $sLe^{x/a}$ in tumor cells has been attributed to elevated levels of α1,3-fucosyltransferase-7 (FUT7), which has also been shown to correspond with increased malignancy in lung cancer patients (161). In addition, overexpression of α1,3-fucosyltransferase-3 and -6 in metastatic prostate cancer cells correlated with higher sLe^x levels and more metastasis that was dependent on E-selectin-mediated recruitment to distant sites (169, 194). Genes encoding for FUT3, FUT4, and ST3GAL6 enzymes that are involved in sLe^x synthesis were significantly

increased in breast cancers and correlated with metastasis to the bone where sLe^x receptor E-selectin is constitutively expressed (195). Inflammatory cytokines might also be involved in sLe^x production. TNF-α enhanced motility and invasion properties of prostatic cancer cells were associated with selective upregulation of genes related to sLe^x synthesis (196). Studies analyzing prostate and pancreatic cancer cell homing into bone showed that E-selectin-mediated adhesion is dependent on enhanced α1,3-fucosyltransferase, FUT3, FUT6, and FUT7 activity (197, 198). Consequently, down-regulation of α1,3-fucosyltransferase activity dramatically reduced prostate cancer incidence. However, there is also the possibility that selectin-mediated activation of either tumor cells or the tumor microenvironment further promote inflammation that is a hallmark of cancer progression.

P-SELECTIN

The association between circulating cancer cells, platelets, and formation of tumor microemboli is widely accepted (199–202). Many studies showed that platelets enhance hematogenous dissemination, intravascular tumor-cell survival, and metastasis (203–206). However, the major mechanism of platelet-adhesion to tumor cells has been found to be mediated by platelet P-selectin (6). Platelet-tumor cell interactions were significantly reduced in P-selectin deficient mice, and consequently attenuation of metastasis was observed. Enzymatic removal of carcinoma mucins carrying selectin ligands from tumor cells prior to tail vein injection resulted in attenuated metastasis comparable to the absence of P-selectin (158, 203). In addition, endothelial P-selectin-mediated interactions also contributed to metastasis indicating that both platelet and endothelial P-selectin promote early events during tissues colonization (11, 207). Another study shows that platelets promote lung metastasis of B16F1 melanoma and 4T1.2 breast cancer cells (208). Platelet depletion resulted in a significant reduction of lung metastasis when compared to NK cell depleted animals, indicating an additional pro-metastatic function of platelets. These findings are in agreement with a direct effect of platelet–tumor-cell interactions that promotes the metastatic behavior of tumor cells (209). Taken together, P-selectin-mediated interactions significantly contribute to the early steps of metastasis when tumor cells are in circulation.

L-SELECTIN

L-selectin binds to a variety of tumor cells and contributes to metastasis (167, 210). Intravenous injection of human and murine tumor cells in L-selectin deficient mice resulted in reduced recruitment of leukocytes and subsequently attenuated metastasis that confirmed the active role of L-selectin-mediated interaction in this process (11, 13). Metastasis was further attenuated in P- and L-selectin double deficient mice providing evidence that both selectins synergistically contribute to metastasis (11). In addition, the enhanced expression of selectin ligands around the metastatic tumor cells was detected with L-selectin chimera, which correlated with the recruitment of leukocytes (13). These findings indicated that L-selectin is either responsible for recruitment of leukocytes or their interactions within the metastatic microenvironment. Enhanced presence of inflammatory cells, primarily myeloid-derived cells, in the tumor microenvironment is usually associated with tumor growth and metastatic dissemination (211, 212). Thus, L-selectin represents a potential facilitator of myeloid cell recruitment to metastatic sites and thereby promotes early steps of metastasis, e.g., tumor-cell extravasation (13, 213). During inflammation, leukocyte interaction with the endothelium results in induced vascular permeability. However, whether L-selectin promotes metastasis through a direct engagement with selectin ligands on tumor cells or rather mimics inflammatory-like reaction accompanying the process of tumor-cell seeding in distant organs remains to be determined.

E-SELECTIN

E-selectin has been the first selectin intensively studied in context of metastasis (9, 10). The original hypothesis was that E-selectin mediates metastatic dissemination to distant organs through binding to ligands on tumor cells, similarly to leukocyte adhesion during inflammation (3). Numerous studies provided evidence that tumor cells expressing selectin ligands adhere to activated endothelium under flow condition *in vitro* (157, 168, 181). While different E-selectin ligands were linked to enhanced metastasis, the majority of them belong to the mucin type molecules. Despite the observation of increased primary tumor growth in selectin deficient mice, which seems to be linked to reduced anti-tumorigenic infiltration of immune cells (214), there is accumulating evidence that E-selectin promotes cancer metastasis in animal models. Enhanced E-selectin expression was observed in the liver during metastatic colonization and the down-regulation of E-selectin resulted in attenuation of metastasis (215, 216). Metastasis was redirected to the E-selectin overexpressing liver using experimental lung metastasis model, which provided direct evidence for involvement of E-selectin in facilitation of tumor-cell seeding (217). Accordingly, experimental liver metastasis of human colon carcinoma cells was also E-selectin-dependent (218). However, experimental lung metastasis of human colon adenocarcinoma cells remained unchanged in E-selectin deficient mice (219). On contrary, spontaneous metastasis of human breast cancer cells to the lungs was significantly attenuated in E-selectin-deficient mice (220). Interestingly, Hiratsuka et al. showed that factors secreted from primary tumors can activate endothelial focal adhesion kinase and E-selectin expression in the lung vasculature and thereby induce the formation of permissible sites for metastasis (221). Enhanced homing of metastatic tumor cells to these sites was observed and was associated with metastasis. These observations indicate that primary tumors can actively form a distant metastatic niche and upregulate expression of cell adhesion molecules involved in tumor cell-endothelial interactions. In conclusion, there is convincing evidence that endothelial E-selectin facilitates metastasis by enabling tumor-cell adhesion to vasculature. Nevertheless, the exact mechanism of E-selectin facilitation of metastasis remains to be defined.

CARCINOMA MUCINS AS INITIATORS OF CANCER-RELATED PROTHROMBOTIC ACTIVITY

Altered cancer glycosylation is not reflected only on cell-surface molecules, but aberrantly glycosylated proteins are detected in the circulation (26). Antibodies raised against tumor cells, were shown to specifically recognize glycan structures, e.g., sLe^a, which

are currently used for cancer diagnostics (45). The presence of carcinoma mucins (e.g., CA-125, CA19-9), which are shedded from tumors, are routinely used as serum tumor markers in diagnosis of cancer. Besides, efficient binding of recombinant soluble selectin to carcinoma mucins has been observed (158, 222). Increased thromboembolism is a recognized complication in various carcinomas, particularly mucinous carcinomas, however; there are several pathologic mechanisms likely to be involved (7). Idiopathic thromboembolism, which is frequently associated with occult carcinomas, belongs to the Trousseau syndrome. Recent studies provided evidence that intravenous injection of carcinoma mucins carrying selectin ligands into mice resulted in generation of platelet-rich microthrombi (222). This pathology was markedly diminished in P-selectin or L-selectin deficient mice. Interestingly, carcinoma mucins could not activate platelets and thereby could not generate microthrombi in mice lacking PSGL-1 (223). Carcinoma mucins initiated thrombosis only in the presence of platelets that induced release of cathepsin G from neutrophils through a selectin-dependent, reciprocal activation of neutrophils and platelets. Taken together, carcinoma mucins carrying selectin ligands in blood circulation may serve as initiators of thrombi formation observed in cancer patients.

SELECTINS SHAPE THE METASTATIC MICROENVIRONMENT

There is accumulating evidence that selectins facilitate heterotypic interactions between tumor cells and blood components, including the endothelium and thereby promote tumor-cell seeding, survival and extravasation (8, 9, 224). When circulating tumor cells arrest in the microvasculature of distant organs, early on markers of endothelial cell activation and inflammation, including E-selectin, were upregulated in experimental lung and liver metastasis models (219, 225–228). Enhanced E-selectin expression was detected also in the metastatic lungs using a spontaneous metastatic model with Lewis lung carcinoma (219, 221). Consequently, inhibition of endothelial activation and/or E-selectin function attenuated metastasis (227, 229). Endothelial activation caused by factors derived from primary tumor or from arrested tumors in the vasculature promoted selectin-mediated interactions and formation of a permissive microenvironment within the vasculature prior to tumor-cell extravasation (11, 13, 213). Tumor-cell glycan-induced and P-selectin-dependent endothelial activation resulted in enhanced expression of E-selectin and vascular cell adhesion molecule 1 (VCAM-1) and promoted lung colonization and metastasis (213). In addition, elevated production of chemokine CCL5 contributed to the recruitment of monocytes. Accordingly, endothelial VCAM-1 expression was induced by tumor-cell embolus that resulted in increased recruitment of myeloid cells supporting metastasis (225). Recruitment of inflammatory cells, especially myeloid-derived cells, is strongly associated with enhanced metastatic colonization that is at least partially dependent on L-selectin (12, 13, 213, 230–232). Taken together, the selectin-mediated interactions play a critical role during the establishment of metastasis that is co-initiated by aberrant glycans on tumor cells in circulation. Whether tumor glycans only initiate the inflammatory-like cascade leading to metastasis or have further function in shaping this process remains to be defined.

CONCLUSION AND PERSPECTIVES

Cancer-associated aberrant glycosylation has been identified in virtually every type of cancer. Expression of cancer-specific glycan epitopes represents a great opportunity to explore them for diagnostics and potentially specific targeting of tumors. Considering that genes only indirectly regulate glycan formation, it is still puzzling that glycan epitopes have been consistently validated as cancer markers. Based on the broad expression and high specificity for cancer tissues, T antigen is currently explored as a potential target for the development of cancer diagnostics and immunotherapeutics (16, 233). Since the expression of sTn antigens on the majority of tumors correlated with poor prognosis, the sTn antigen has become a target for cancer vaccine (58, 61). Administration of sTn disaccharide conjugate to highly immunogenic protein induced antibodies against sTn and showed protective effects in a mouse model of breast cancer (234). Although a randomized phase III clinical trial using the same sTn vaccine did not improve overall survival, patients with high titer against the sTn had significantly prolonged overall survival (235).

The accumulating knowledge about the function of lectin–tumor-cell glycan interactions in cancer will open ways for new approaches to interfere with cancer progression. However, the exploitation of such therapeutic opportunities requires a comprehensive knowledge about the underlying mechanisms of lectin-mediated interactions. Nevertheless, the role of selectins in cancer progression has been extensively investigated in number of preclinical models and the mechanism at least partially characterized (9). Clearly, further studies in the exact mechanism of action are still required, but selectin inhibition in cancer has been inadvertently clinically tested in cancer patients treated with antithrombotic therapies (236). Unfractionated heparin as well as low molecular weight heparin has a strong P- and L-selectin inhibitory activity at clinically relevant concentrations. Retrospective analysis of clinical studies revealed that apart from antithrombotic activity, heparin improved survival of cancer patients especially in patients with early stage disease. Still, prospective and well-designed clinical study remains to be performed. Similarly, development of highly specific ligand probes for siglecs (e.g., Siglec-2) revealed the ability to target siglec-expressing cells (94). Further investigations are required for deciding whether glycan-specific targeting of lectins involved in cancer modulation (e.g., siglec, selectins, or galectins) or rather development of glycan-specific targeting of tumor cells represents the right approach for the treatment of cancer. The cell-surface presentation of unique glycan epitopes makes them an "ideal" candidate for targeting since they are both specific and therapeutically accessible. Future studies need to validate the therapeutic potential in clinically relevant experimental models prior to clinical evaluation.

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